PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 April 2001 (26.04.01)	
International application No. PCT/IL00/00330	**Applicant's or agent's file reference** 20101
International filing date (day/month/year) 07 June 2000 (07.06.00)	**Priority date** (day/month/year) 10 June 1999 (10.06.99)
Applicant SHANI, Ziv et al	

1. The designated Office is hereby notified of its election made:

[X] *in the demand* filed with the International Preliminary Examining Authority on:

27 December 2000 (27.12.00)

[ ] *in a notice effecting later election* filed with the International Bureau on:

2. The election [X] was

[ ] *was not*

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Claudio Borton
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREAT.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
AMENDMENTS OF THE CLAIMS

(PCT Rule 62 and
Administrative Instructions, Section 417)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as International Preliminary Examining Authority

Date of mailing (day/month/year)	
26 April 2001 (26.04.01)	
International application No.	International filing date (day/month/year)
PCT/IL00/00330	07 June 2000 (07.06.00)

Applicant

CBD TECHNOLOGIES LTD. et al

The International Bureau hereby informs the International Preliminary Examining Authority that no amendments under Article 19 have been received by the International Bureau (Administrative Instructions, Section 417).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Claudio Borton

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

G.E. EHRLICH (1995) LTD.
17th Floor
28 Bezalel Street
52521 Ramat Gan
ISRAËL

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
20 July 2000 (20.07.00)	
Applicant's or agent's file reference	**International application No.**
20101	PCT/IL00/00330

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

CBD TECHNOLOGIES LTD. et al (for all designated States except US)
SHANI, Ziv et al (for US)

International filing date	:	07 June 2000 (07.06.00)
Priority date(s) claimed	:	10 June 1999 (10.06.99)
Date of receipt of the record copy by the International Bureau	:	23 June 2000 (23.06.00)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EE,ES, FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD, MG,MK,MN,MW,MX,MZ,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ, VN,YU,ZA,ZW

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [ ] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Aino Metcalfe

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 20101	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IL00/00330	International filing date *(day/month/year)* 07 JUNE 2000	Priority date *(day/month/year)* 10 JUNE 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant SHANI, ZIV		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27 DECEMBER 2000	27 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ASHWIN MEHTA Telephone No. (703) 306-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. 12-25

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-11	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-11	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-11	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-11 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of expressing in, and isolating from, a cellulose binding domain-fusion proteins, comprising expressing the protein such that it does not contact cells walls, and homogenizing the plant so that the cellulose binding domain of the fusion protein binds the cellulosic matter of the plant.

Claims 1-11 meet the criteria set out in PCT Article 33(4) because it has the industrial applicability of using plants for the production of useful proteins.

---------- NEW CITATIONS ----------

KLEIN et al. Transformation Of Microbes, Plants And Animals By Particle Bombardment. Biotech. March 1992, Vol. 10, pages 286-291, see whole document.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Amended claim 1 and claims 2-5 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s):

The claims are missing step(s). Claim 1 is drawn to a method of producing and isolating a protein of interest. However, the last step of the claims result in a fusion protein cellulosic matter complex, not the protein of interest.

Further regarding claim 3- claim 3 states that the fusion protein cellulosic matter complex is the final product. However, line 2 of claim 1 indicates that the protein of interest should be the final product.

In the amendment submitted 20 July 2001 Applicants argue that the method is directed at producing several alternative final products, and the common denominator of each is the protein of interest. However, the preamble of claim 1, even as amended, clearly calls for isolation of the protein of interest, and not for any alternative final product.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12N 5/04, 15/09, 15/62, 15/64, 15/67, 15/82, 15/90; A01H 5/00 and US Cl.: 435/69.1, 320.1, 410, 419, 468; 800/278, 287, 288, 295, 298

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-52, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) NONE, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Pages 53-54, filed with the letter of 20 July 2001

This report has been drawn on the basis of the drawings,
page(s) 1-11, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: G.E. EHRLICH (1995) LTD.
28 BEZALEL STREET, 17TH FLOOR
52 521 RAMAT GAN
ISRAEL

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 28 NOV 2000
Applicant's or agent's file reference 20101	**FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/IL00/00330	International filing date *(day/month/year)* 07 JUNE 2000
Applicant SHANI, ZIV	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ASHWIN MEHTA Dorithea Lawrence for Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)★ *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 20101	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/IL00/00330	International filing date *(day/month/year)* 07 JUNE 2000	(Earliest) Priority Date *(day/month/year)* 10 JUNE 1999
Applicant SHANI, ZIV		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: GAL EHRLICH
C/O ANTHONY CASTORINA
2001 JEFFERSON DAVIS HIGHWAY
SUITE 207
ARLINGTON, VA 22202

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

	Date of Mailing *(day/month/year)* 07 NOV 2001
Applicant's or agent's file reference 20101	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/IL00/00330	07 JUNE 2000	10 JUNE 1999

Applicant
SHANI, ZIV

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ASHWIN MEHTA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

#8 PB 2/22/02

09/980,469 Corrected

PATENT COOPERATION TREATY

PCT

REC'D 10 APR 2002
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 20101	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IL00/00350	International filing date *(day/month/year)* 07 JUNE 2000	Priority date *(day/month/year)* 10 JUNE 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant SHANI, ZIV		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

CORRECTED VERSION

Date of submission of the demand	Date of completion of this report
27 DECEMBER 2000	27 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ASHWIN MEHTA Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

I. Basis of the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached), as originally filed
pages ____, filed with the demand
pages ____, filed with the letter of ____

☒ the claims:
pages (See Attached), as originally filed
pages ____, as amended (together with any statement) under Article 19
pages ____, filed with the demand
pages ____, filed with the letter of ____

☒ the drawings:
pages (See Attached), as originally filed
pages ____, filed with the demand
pages ____, filed with the letter of ____

☒ the sequence listing part of the description:
pages (See Attached), as originally filed
pages ____, filed with the demand
pages ____, filed with the letter of ____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. 12-25

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-11	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-11	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-11	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-11 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of expressing in, and isolating from, a cellulose binding domain-fusion proteins, comprising expressing the protein such that it does not contact cells walls, and homogenizing the plant so that the cellulose binding domain of the fusion protein binds the cellulosic matter of the plant.

Claims 1-11 meet the criteria set out in PCT Article 33(4) because it has the industrial applicability of using plants for the production of useful proteins.

NEW CITATIONS

KLEIN et al. Transformation Of Microbes, Plants And Animals By Particle Bombardment. Biotech. March 1992, Vol. 10, pages 286-291, see whole document.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Amended claim 1 and claims 2-5 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s):

The claims are missing step(s). Claim 1 is drawn to a method of producing and isolating a protein of interest. However, the last step of the claims result in a fusion protein cellulosic matter complex, not the protein of interest. Further regarding claim 5- claim 5 states that the fusion protein cellulosic matter complex is the final product. However, line 2 of claim 1 indicates that the protein of interest should be the final product.

In the amendment submitted 20 July 2001 Applicants argue that the method is directed at producing several alternative final products, and the common denominator of each is the protein of interest. However, the preamble of claim 1, even as amended, clearly calls for isolation of the protein of interest, and not for any alternative final product.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IL00/00330

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII **Sheet 10**

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12N 5/04, 15/09, 15/62, 15/64, 15/67, 15/82, 15/90; A01H 5/00 and US Cl.: 435/69.1, 320.1, 410, 419, 468; 800/278, 287, 288, 295, 298

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-52, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) NONE, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Pages 53-54, filed with the letter of 20 July 2001

This report has been drawn on the basis of the drawings,
page(s) 1-11, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

PCT/IL00/00330
IPEA/US 20 JUL 2001

WHAT IS CLAIMED IS:

1. A process of producing a protein of interest in a plant, plant derived tissue or cultured plant cells and of isolating the protein from the plant, plant derived tissue or cultured plant cells, the process comprising the steps of:

(a) providing a plant, a plant derived tissue or cultured plant cells expressing a fusion protein including the protein of interest and a cellulose binding peptide being fused thereto, said fusion protein being compartmentalized within cells of said plant, plant derived tissue or cultured plant cells, so as to be sequestered from cell walls of said cells of said plant, plant derived tissue or cultured plant cells;

(b) homogenizing said plant, plant derived tissue or cultured plant cells, so as to bring into contact said fusion protein with a plant derived cellulosic matter of said plant, plant derived tissue or cultured plant cells, to thereby effect affinity binding of said fusion protein via said cellulose binding peptide to said cellulosic matter, thereby obtaining a fusion protein cellulosic matter complex; and

(c) isolating said fusion protein cellulosic matter complex.

2. The process of claim 1, further comprising the step of:

(d) washing said fusion protein cellulosic matter complex, thereby removing endogenous plant proteins and other plant material therefrom.

3. The process of claim 2, further comprising the step of:

(e) collecting said fusion protein cellulosic matter complex as a final product of the process.

4. The process of claim 2, further comprising the step of:

(e) exposing said fusion protein cellulosic matter complex to conditions effective in dissociating said fusion protein from said cellulosic matter; and

(f) isolating said fusion protein, thereby obtaining an isolated fusion protein.

PCT/IL00/00330
IPEA/US 20 JUL 2001

5. The process of claim 4, wherein said conditions effective in dissociating said fusion protein from said cellulosic matter are selected from the group consisting of basic conditions, denaturative conditions and affinity displacement conditions.

6. The process of claim 4, further comprising the step of:

(g) exposing said isolated fusion protein to conditions effective in digesting said fusion protein so as to release said protein of interest from said fusion protein, thereby obtaining a released protein of interest.

7. The process of claim 6, wherein said conditions effective in digesting said fusion protein so as to release said protein of interest therefrom are selected from the group consisting of proteolysis effected via a protease and proteolysis effected under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

8. The process of claim 6, further comprising the step of:

(h) isolating said released protein of interest.

9. The process of claim 2, further comprising the step of:

(e) exposing said fusion protein cellulosic matter complex to conditions effective in digesting said fusion protein so as to release said protein of interest therefrom, thereby obtaining a released protein of interest.

10. The process of claim 9, wherein said conditions effective in digesting said fusion protein so as to release said protein of interest therefrom are selected from the group consisting of proteolysis effected via a protease and proteolysis effected under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

11. The process of claim 9, further comprising the step of:

(f) isolating said released protein of interest.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purpose of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When ? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

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Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase*

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The aim of the work described in this paper was to characterize the tubers of potato (*Solanum tuberosum* var. Prairie) plants that had been transformed with the *Escherichia coli* ADPglucose pyrophosphorylase (EC 2.7.7.27) gene, *glgC-16*, under the control of a patatin promoter. Over 30 lines of transformed plants with increased ADPglucose pyrophosphorylase activity were obtained. The tubers of six of these lines were compared with those of control plants expressing the gene for β-glucuronidase. The average increase in pyrophosphorylase activity was 200%, and the highest was 400%. Western immunoblotting of tuber extracts showed that the amounts of glgC-16 protein were linearly related to the extractable activity of the ADPglucose pyrophosphorylase. Cell fractionation studies showed that the increased activity of the pyrophosphorylase in the *glgC-16* tubers had a similar intracellular location, the amyloplast fraction, to that found in the control tubers. No pleiotropic changes in the maximum catalytic activities of the following enzymes could be detected in the *glgC-16* tubers: sucrose synthase, fructokinase, UDPglucose pyrophosphorylase, phosphofructokinase, soluble starch synthase, starch branching enzyme, phosphoglucomutase and alkaline inorganic pyrophosphatase. The *glgC-16* tubers are held to be suitable for the study of the role of ADPglucose pyrophosphorylase in the control of starch synthesis.

INTRODUCTION

Although there is appreciable evidence for a central role for ADPglucose pyrophosphorylase (EC 2.7.7.27) in the regulation of starch synthesis in chloroplasts, its role in amyloplasts is less well understood [1–3]. In an attempt to remedy this deficiency we have studied the metabolism of potato tubers in which the amount of ADPglucose pyrophosphorylase has been increased by transformation with the *Escherichia coli* gene *glgC-16*. The latter encodes a mutant form of ADPglucose pyrophosphorylase that shows a reduced response to allosteric effectors [4]. This gene was expressed specifically in tubers by placing it under the control of a patatin promoter and was targeted to the plastid by using a transit peptide from the small subunit of ribulosebisphosphate carboxylase. As controls we used tubers from plants that were expressing the gene for β-glucuronidase (GUS-control). We chose to transform the tubers with a foreign gene in order to reduce the risk of co-suppression and because it is easier to manipulate the *E. coli* enzyme, which is a homotetramer, than the potato enzyme, which is a heterotetramer. We knew that a similar approach had been taken by Stark et al. [5], but argue that their data cannot be used for control analysis because no measurements of flux to starch or of the activity of ADPglucose pyrophosphorylase were reported.

Our argument is that if ADPglucose pyrophosphorylase plays a key role in the control of starch synthesis in potato tubers, then increasing the amount of this enzyme as just described should lead to an increased rate of synthesis. The availability of a range of transgenic tubers with differing activities of the pyrophosphorylase might allow calculation of the control coefficient of this enzyme in respect of starch synthesis. Our approach depends crucially upon adequate characterization of the transgenic plants [6]. The aim of the work described in the present paper was to provide that characterization.

The first essential was to determine the extent to which the maximum catalytic activity of ADPglucose pyrophosphorylase had been altered. We stress that it is the activity of an enzyme that is paramount in determining its role in control, and that measurements of its mRNA or even the total amount of enzyme protein, though important, are not satisfactory substitutes as they may not be proportional to enzyme activity. The second essential was to check whether the increased pyrophosphorylase activity had the same location as the native enzyme. Finally it was essential to investigate whether any change in ADPglucose pyrophosphorylase activity had been accompanied by pleiotropic changes in other enzymes involved in starch synthesis. In making these checks we took particular care to avoid the pitfalls inherent in measuring enzymes in extracts of potato tubers [7]. For each enzyme we optimized the assay and checked that losses of activity did not occur during extraction and assay. We did this by determining the recovery of samples of pure enzymes added to the tissue sample before extraction.

EXPERIMENTAL

Materials

Except where stated otherwise, substrates and enzymes were from Boehringer-Mannheim U.K. (Lewes, E. Sussex, U.K.). Glucose 1,6-bisphosphate, kanamycin A and ampicillin were from Sigma Chemical Co. (Poole, Dorset, U.K.). Radiochemicals and the Amersham ECL immunodetection kit were from

Abbreviation used: GUS-control plant, plant expressing the gene for β-glucuronidase.

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* On 3 October 1996, Professor Tom ap Rees was tragically killed while cycling home. We dedicate this paper to his memory.

Amersham International (Aylesbury, Bucks., U.K.). ADPglucose pyrophosphorylase from *E. coli glgC-16* and polyclonal antibodies to it were kindly supplied by Advanced Technologies (Cambridge) Ltd. (Cambridge, U.K.). Antibodies were produced as follows. The *glgC-16* coding sequence [8] was cloned into the pGex vector (Pharmacia Biotech), and the vector was expressed in *E. coli* according to the manufacturer's instructions. The *glgC-16* gene product was purified by absorption on to glutathione–L-Sepharose equilibrated with 50 mM Tris, pH 8.0, and 150 mM NaCl. After washing the resin with equilibration buffer, the protein was eluted in 50 mM Tris, pH 8.0, 5 mM glutathione and 150 mM NaCl. Antibodies to the purified fusion protein were obtained from New Zealand white rabbits as described by Burrell et al. [8a].

Plants

The work was carried out with tubers from GUS-control and *glgC-16*-transformed plants of *Solanum tuberosum* var. Prairie. These plants were generously supplied by Advanced Technologies (Cambridge) Ltd. The GUS-control plants were produced by making the plasmid pFW4101 from pBin19 [9] with a patatin promoter made from two genomic clones, *ps3* and *ps27* [10], and the coding sequence for β-glucuronidase. This plasmid was introduced into *Agrobacterium tumefaciens*, strain C58, which was then used to transform potato leaf discs as described by Blundy et al. [10]. Kanamycin-resistant cells were used to regenerate shoots *in vitro* on Murashige and Skoog's medium [11]. Plants expressing the *E. coli glgC-16* gene were produced in the same way, except that the leaf discs were inoculated with *Agrobacterium* that contained plasmid pFW4173. The latter was made from plasmid pFW4101, with the β-glucuronidase coding sequence being replaced by that of a ribulose bisphosphate carboxylase transit peptide and the *glgC-16* gene [8]. The regenerated shoots were grown at 22 °C in a 16-h photoperiod and a quantum irradiance of 159 μE/s per m². Microtubers were induced by transferring the shoots to Murashige and Skoog's medium [11] that contained 2.5 mg/l kinetin and 4.7 mg/l ancymidol, and growing at 22 °C in the dark for 4–6 weeks. To obtain tubers, shoots were planted in Fisons Levingtons F1 compost in pots of 63 mm diameter and grown in a greenhouse at 16–20 °C in a 16-h photoperiod of daylight supplemented with artificial light that gave a minimum quantum irradiance of 125 μE/s per m². After 1 week in a propagator the plants were transplanted into a mixture of Perlite and Levingtons M2 compost (1:2, v/v) in 130 mm diameter pots and grown for 2–3 months under the conditions just described. Plant lines were propagated clonally by planting cuttings of shoot tips as we have described above. Experiments were carried out with tubers of 10–50 g fresh weight either immediately after harvest or after a brief period of storage at 4 °C.

Enzyme assays

Tubers were cut into 2 mm-thick slices, which were immediately freeze-clamped and ground to a fine powder in liquid N_2 with a pestle and mortar. The frozen powder was immediately transferred to −180 °C and stored at this temperature for up to 2 months before use. To assay enzyme activity, 1 g of the frozen powder was resuspended at 4 °C in 5 ml of extraction medium [100 mM Hepes, pH 7.5, 10 mM EDTA, 5 mM dithiothreitol, 0.5% (w/v) BSA] that contained 0.1% (w/v) polyvinylpyrrolidone. After 5 min the suspension was centrifuged at 10000 *g* for 5 min at 4 °C and the supernatant was desalted by passage through a column (5 cm × 1.5 cm) of PD-10 Sephadex (G-25M) equilibrated with extraction medium, and then assayed.

Unless otherwise stated, enzymes were assayed at 25 °C in the following 1.0 ml reaction mixtures as described in the accompanying references. ADPglucose pyrophosphorylase (EC 2.7.7.27): 40 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 0.4 mM NAD^+, 0.024 mM glucose 1,6-bisphosphate, 1.5 mM ADPglucose, 2 mM $Na_4P_2O_7$, 4 units of phosphoglucomutase and 1.4 units of glucose-6-phosphate dehydrogenase (NAD^+-specific from *Leuconostoc mesenteroides*) [12]. Soluble starch synthase (EC 2.4.1.21): 150 mM Bicine, pH 8.4, 400 mM sodium citrate, 0.1 mg of potato amylopectin and 1.4 mM ADP[U-^{14}C]glucose (5.3 kBq/μmol) in 200 μl at 30 °C [13]. Starch branching enzyme (EC 2.4.1.18): 100 mM sodium citrate, pH 6.0, 1 mM AMP, 50 mM [U-^{14}C]glucose 1-phosphate (1.5 kBq/μmol) and 0.02 unit of glycogen phosphorylase from rabbit muscle in 50 μl at 30 °C [14]. Alkaline inorganic pyrophosphatase (EC 3.6.1.1): 50 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$ and 1.5 mM $Na_4P_2O_7$ in 200 μl [15]. Sucrose synthase (EC 2.4.1.13): 100 mM Hepes, pH 7.5, 4 mM $MgCl_2$, 0.4 mM UDPglucose, 1 mM phosphoenolpyruvate, 10 mM fructose, 0.2 mM NADH, 10 units of pyruvate kinase and 2 units of lactate dehydrogenase [16]. UDPglucose pyrophosphorylase (EC 2.7.7.9): 80 mM glycylglycine, pH 8.0, 1 mM $MgCl_2$, 10 μM glucose 1,6-bisphosphate, 0.4 mM NAD^+, 0.8 mM UDPglucose, 1 mM $Na_2H_2P_2O_7$, 4 units of phosphoglucomutase and 1.4 units of glucose-6-phosphate dehydrogenase (NAD^+-dependent) [17]. 6-Phosphofructokinase (EC 2.7.1.11): 100 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM ATP, 1 unit of aldolase, 10 units of triosephosphate isomerase and 1.3 units of glycerol-3-phosphate dehydrogenase [18]. Phosphoglucomutase (EC 5.4.2.2): 50 mM Hepes, pH 7.6, 1 mM $MgCl_2$, 0.25 mM glucose 1-phosphate, 0.024 mM glucose 1,6-bisphosphate, 0.4 mM NAD^+ and 1.5 units of glucose-6-phosphate dehydrogenase (NAD^+-specific) [19]. Fructokinase (EC 2.7.1.4): 100 mM Hepes, pH 8.2, 4 mM $MgCl_2$, 0.2 mM fructose, 2.5 mM ATP, 0.3 mM NAD^+, 1 unit of glucose-6-phosphate dehydrogenase (NAD^+-dependent) and 5 units of glucose-6-phosphate isomerase [20]. Alcohol dehydrogenase (EC 1.1.1.1): 50 mM Hepes, pH 7.8, 2 mM NAD^+ and 150 mM ethanol [21].

Immunodetection of the glgC-16 protein

Portions of the desalted extract used for the enzyme assays were heated to 100 °C for 5 min and were then subjected to discontinuous SDS/PAGE [22]. The separated proteins were electroblotted on to poly(vinylidene difluoride) membranes which were incubated first with polyclonal antiserum raised against the glgC-16 protein, and then with anti-rabbit IgG conjugated to horseradish peroxidase (Amersham International). Bound antibody was detected with the Amersham ECL immunodetection kit.

Cell fractionation

Amyloplasts were separated from the cytosol of potato tubers by taking a core (1.4 cm × 6 cm) of tissue (10 g fresh weight) longitudinally through a tuber. The core was cut into discs 1–2 mm thick with a razor blade into 15 ml of 50 mM Hepes, pH 7.6, 1 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM KCl, 0.2% (w/v) BSA and 5 mM dithiothreitol (homogenization medium). This first 15 ml of homogenization medium was discarded, and the discs were washed twice more with 10 ml lots of medium. Next the discs were chopped very finely with razor blades in 10 ml of homogenization medium for no more than 5 min. The resulting suspension was filtered through four layers of muslin that had been soaked in homogenization medium. The filtrate (10 ml) was layered on to a stepped gradient that consisted

of 5 ml of 60 % (w/v) Nycodenz overlaid with 10 ml of 1 % (w/v) Nycodenz. The Nycodenz was dissolved in homogenization medium. The gradient was allowed to stand for 2 h, by which time a white band, referred to as amyloplasts, had formed at the interface between the 1 % and 60 % Nycodenz. The rest of the homogenate remained above the 1 % Nycodenz (the supernatant fraction). These two fractions were completely removed and assayed for the appropriate enzymes. The whole process of homogenization and fractionation was carried out at 4 °C.

Before assay of enzyme activity in the fractions or the unfractionated homogenate, care was taken to rupture any organelles present. For ADPglucose pyrophosphorylase this was done by making the sample 0.1 % (v/v) with respect to Triton X-100. For all other assays the sample was subjected to three cycles of freezing in liquid N_2 and thawing at 37 °C.

Protein was measured with the Bio-Rad assay kit [23], and ^{14}C was determined by liquid-scintillation counting with Optiphase scintillation fluid.

RESULTS

Increased activity of ADPglucose pyrophosphorylase in transgenic tubers

Initial experiments indicated that tubers from transformed line no. 123 had enhanced ADPglucose pyrophosphorylase activity. We used extracts of mature tubers of this line that had been stored for 8 weeks to authenticate our assay procedure. The concentration of each component in, and the pH of, the assay were optimized to give the values listed in the Experimental section. Activity was shown to be linearly related to the volume of extract used and, after an initial lag, to time. As expected from the work of Merlo et al. [24], we found significant variation in the activity of ADPglucose pyrophosphorylase in extracts of different parts of the same tuber. To combat this heterogeneity within tubers, we took the complete tuber as our basic sample for analysis. The tuber was sliced and the slices were instantly freeze-clamped and ground to a homogeneous powder in liquid N_2. A subsample of this powder was used for the measurement of enzyme activity. We checked that this procedure did not result in loss of enzyme activity by comparing activity in extracts prepared from the frozen powder and extracts prepared by homogenizing fresh tissue in extraction medium. The native potato ADPglucose pyrophosphorylase and the introduced *E. coli* enzyme respond differently to 3-phosphoglycerate. Thus, in order to compare activities in *glgC*-16 transgenic and GUS-control tubers, we assayed the enzyme in the absence of exogenous 3-phosphoglycerate. This explains why the activities that we report are lower than those found by Merlo et al. [24] and by Geigenberger et al. [25].

We completed recovery experiments to check for losses during extraction of the tissue. In each test we took duplicate samples of powdered freeze-clamped tissue. One sample was extracted and assayed in the usual way, and the other was treated similarly except that a measured activity of pure glgC-16 protein was added to the frozen powder prior to extraction. The added activity was comparable with that present in the untreated sample. Comparison of the activities in the two samples allowed estimation of the recovery of the pure enzyme. Values of 102 ± 10 % and 112 ± 11 % (means $\pm$ S.E.M., $n = 3$) were obtained for *glgC-16* transgenic and GUS-control tubers respectively. The optimized assay was used in all subsequent work.

Microtubers from 37 different lines of transformed shoots were assayed for ADPglucose pyrophosphorylase activity. Values ranged from 120 to 560 nmol/min per g fresh weight. For further studies we selected six lines that represented the range of these

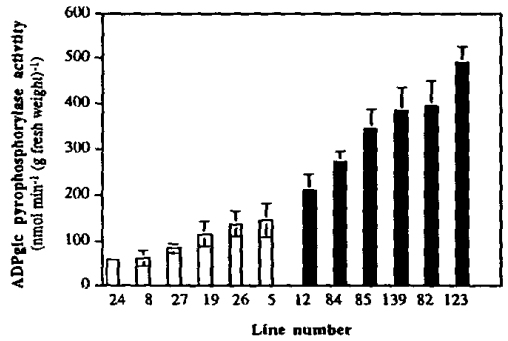

Figure 1 ADPglucose pyrophosphorylase activity in developing tubers from *glgC-16* and GUS-control plants

Developing tubers (10–50 g fresh wt.) were freeze-clamped immediately after harvest from 10-week-old plants. Each value is the mean of estimates from at least five tubers; bars represent S.E.M. □, GUS-control lines, ■, *glgC-16* lines.

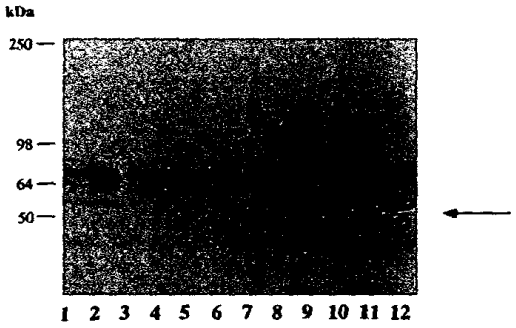

Figure 2 Western blot analysis of glgC-16 protein in extracts of cold-stored tubers from *glgC-16*-transformed and GUS-control plants

Extracts of frozen powders from tubers that had been stored at 4 °C for 3 months were centrifuged and the supernatant fractions desalted prior to analysis by SDS/PAGE and Western blotting. Protein loading was 100 μg per lane. Lanes 1–3, GUS-control line 5; lanes 4–6, *glgC-16* line 12; lanes 7–9, *glgC-16* line 82; lanes 10–12, *glgC-16* line 123. The arrow indicates glgC-16 protein.

activities. First, we compared the maximum catalytic activity of ADPglucose pyrophosphorylase in tubers from 10-week-old plants of each of these lines with that of comparable tubers from six GUS-control lines (Figure 1). The activity in each of the *glgC-16* lines was higher than the average activity of the GUS-control lines ($P < 0.05$). The activity in each of the *glgC-16* lines, except for no. 12, was greater than that of the highest value found in any GUS-control line ($P < 0.05$).

We used Western blotting to determine whether the increase in ADPglucose pyrophosphorylase activity in the *glgC-16* lines was paralleled by increases in the amount of the glgC-16 protein. We extracted proteins from tubers that had been stored at 4 °C for 3 months, separated them by SDS/PAGE and treated them with an antibody raised to glgC-16 protein from *E. coli*. The glgC-16 protein has a molecular mass of 50 kDa [26], and was clearly present in tubers from the transformed lines 82 and 123 (Figure 2). The glgC-16 protein could not be detected in tubers of the

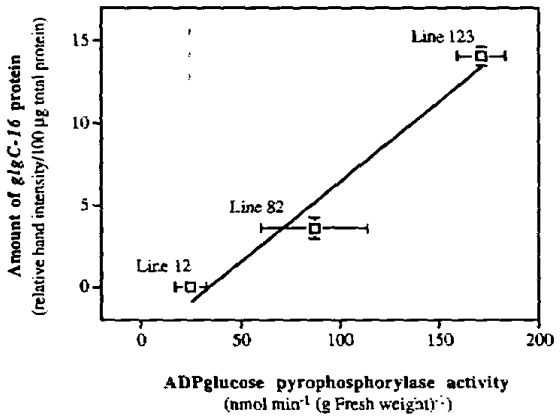

Figure 3 Relationship between ADPglucose pyrophosphorylase activity and the amount of glgC-16 protein in extracts of *glgC-16*-transformed tubers

Cold-stored tubers of *glgC-16* transgenic lines 12, 82 and 123 were freeze-clamped. Extracts of the powders were centrifuged and the supernatant fractions desalted, assayed for enzyme activity and analysed by Western blotting. The density of the bands on the blots was measured by scanning densitometry. Values are the means ± S.E.M. of results from three tubers of each line.

GUS-control line 5, or in tubers of the transformed line 12 which did not have a significantly increased activity of ADPglucose pyrophosphorylase. The antibody also recognized a second protein, of unknown identity, with a molecular mass of approx. 65 kDa. This protein was present in both control and transformed tubers, but is not the tuber ADPglucose pyrophosphorylase, which has a molecular mass of 50 kDa [27].

Qualitative comparison of Figures 1 and 2 suggests that the activity of ADPglucose pyrophosphorylase rose in proportion to the amount of glgC-16 protein present. We prepared extracts from three different *glgC-16* transgenic lines and measured both enzyme activity and the density of the band corresponding to glgC-16 protein on Western blots, by scanning densitometry, for each extract. Figure 3 shows that there is a linear relationship between our estimates of enzyme activity and our estimates of the amount of enzyme protein. The plot intersects the *x*-axis at a value that is equivalent to the activity of the enzyme in cold-stored GUS-control tubers.

Intracellular location of ADPglucose pyrophosphorylase

For the relatively few non-photosynthetic tissues for which data are available, the evidence strongly suggests that ADPglucose pyrophosphorylase is largely, if not exclusively, confined to the plastid. However, there is some evidence that there is a cytosolic ADPglucose pyrophosphorylase in some cereal endosperms [28]. No data are available for potato tubers. In order to investigate whether the increased activity of the pyrophosphorylase in the *glgC-16*-transformed tubers had the same intracellular location as the pyrophosphorylase in the control tubers, we made preparations of amyloplasts from each type of tuber (Table 1). We used alkaline inorganic pyrophosphatase as a marker for amyloplasts [29] and alcohol dehydrogenase as a marker for the cytosol. Our fractionation procedure yielded only two fractions, a particulate amyloplast fraction and a supernatant. For each enzyme studied the sum of the activities recovered in the amyloplast fraction and the supernatant was similar to the activity found in the unfractionated homogenate. Thus our data are not seriously affected by losses during analysis. The activities of the marker enzymes were comparable in the extracts of the GUS-control and *glgC-16* transgenic tubers. As expected, the latter showed higher activity of ADPglucose pyrophosphorylase. We obtained a modest but significant yield of amyloplasts that were essentially free of cytosolic contamination. The distribution of the marker enzymes in preparations from GUS-control tubers was not significantly different from that in preparations from *glgC-16*-transformed tubers. Of most importance is the fact that the distribution of ADPglucose pyrophosphorylase followed that of the amyloplast marker in the preparations of both the GUS-control and *glgC-16* tubers.

Search for pleiotropic effects

At no stage during development could we detect any phenotypic difference between the *glgC-16*-transformed plants and the GUS-control plants.

We investigated whether the increased activity of ADPglucose pyrophosphorylase in the *glgC-16* plants was accompanied by changes in the maximum catalytic activities of other enzymes connected with starch synthesis. We checked the assays for each enzyme as described for the assay of ADPglucose pyrophosphorylase. Each assay was optimized. For each enzyme we showed that freeze-clamping the tuber tissue and then extracting the frozen powder gave a similar activity to that found when the tuber tissue was homogenized directly in extraction medium.

Table 1 Activities of ADPglucose pyrophosphorylase in amyloplast preparations from GUS-control and *glgC-16* transgenic tubers

Tubers were from 8-week-old plants and had been stored at 4 °C for 6 weeks. Cores of tuber were chopped with razor blades into extraction medium to give a suspension that was filtered through muslin to produce the unfractionated homogenate. The latter was fractionated on a stepped gradient of Nycodenz to give an amyloplast fraction and a supernatant fraction, and activities in each fraction are expressed as percentages of that in the unfractionated homogenate. Values are means ± S.E.M. of data from fractionations of three different tubers of each type.

Enzyme	Tuber	Activity in homogenate (nmol/min per ml)	Activity recovered in fraction (%)	
			Amyloplasts	Supernatant
Alcohol dehydrogenase	GUS-control	70 ± 8	0.7 ± 0.2	97 ± 1
	glgC-16	75 ± 7	0.9 ± 0.2	102 ± 3
Alkaline pyrophosphatase	GUS-control	67 ± 6	6.9 ± 1.5	100 ± 7
	glgC-16	73 ± 2	7.1 ± 1.5	104 ± 7
ADPglucose pyrophosphorylase	GUS-control	4.5 ± 0.5	7.3 ± 1.1	92 ± 4
	glgC-16	17.9 ± 1.2	8.0 ± 0.8	95 ± 5

Table 2 Estimates of the maximum catalytic activities of enzymes of starch metabolism in GUS-control and *glgC-16* transgenic tubers after harvest from 10-week-old plants

Frozen powder from freeze-clamped tubers was suspended in extraction medium at 4 °C for 5 min and then centrifuged at 10000 *g* for 5 min. The supernatant was assayed for enzyme activities (nmol/min per g fresh wt., except where indicated otherwise). Values are means ± S.E.M. of results from the numbers of tubers shown in parentheses.

	Activity (nmol/min per g fresh wt.)						
		glgC-16 transgenic					
	GUS-control	Line 12	Line 84	Line 85	Line 139	Line 82	Line 123
ADPglucose pyrophosphorylase	100 ± 12 (12)	212 ± 33 (5)	273 ± 24 (5)	345 ± 45 (5)	386 ± 51 (5)	398 ± 53 (5)	493 ± 34 (5)
Alkaline inorganic pyrophosphatase	408 ± 40 (12)	365 ± 27 (3)	397 ± 19 (3)	370 ± 37 (3)	405 ± 48 (3)	444 ± 51 (3)	406 ± 42 (3)
Soluble starch synthase	119 ± 7 (12)	118 ± 36 (4)	164 ± 50 (4)	135 ± 48 (4)	173 ± 56 (4)	142 ± 33 (4)	150 ± 48 (4)
Branching enzyme*	4.1 ± 0.4 (12)	5.1 ± 1.8 (4)	4.5 ± 1.5 (4)	5.4 ± 1.3 (4)	4.0 ± 1.6 (4)	4.9 ± 1.4 (4)	3.7 ± 1.9 (4)
Phosphoglucomutase †	9.2 ± 0.4 (6)	11.4 ± 0.5 (4)	–	–	–	9.4 ± 0.6 (4)	10.2 ± 0.8 (4)
Sucrose synthase	959 ± 129 (18)	674 ± 297 (3)	705 ± 18.3 (3)	1034 ± 282 (3)	956 ± 100 (3)	640 ± 292 (3)	788 ± 371 (3)
Fructokinase	242 ± 17 (18)	214 ± 60 (3)	278 ± 15 (3)	272 ± 23 (3)	208 ± 26 (3)	280 ± 72 (3)	230 ± 33 (3)
Phosphofructokinase	164 ± 10 (12)	161 ± 10 (4)	–	–	–	195 ± 21 (4)	192 ± 18 (4)
UDPglucose pyrophosphorylase†	7.0 ± 0.7 (18)	6.5 ± 1.2 (3)	7.6 ± 1.0 (3)	8.1 ± 0.1 (3)	7.6 ± 1.2 (3)	8.0 ± 1.4 (3)	8.3 ± 0.4 (3)

* Values are 10^{-3} × fold stimulation of the activity of glycogen phosphorylase/g fresh wt.

† Values are μmol/min per g fresh wt.

Recovery experiments were carried out with both *glgC-16*-transformed tubers (line 123) and GUS-control tubers for phosphofructokinase, fructokinase, UDPglucose pyrophosphorylase and phosphoglucomutase. Recoveries of phosphoglucomutase were 79% and 73% respectively for the GUS-control and *glgC-16* tubers. For the remaining enzymes, recoveries were not significantly different from 100%. For soluble starch synthase, branching enzyme and sucrose synthase we carried out recombination experiments, where we measured activity in a sample composed of frozen tuber powder and material from the spadix of *Arum maculatum*, and compared the value obtained with that predicted from measurements made on the separate components of the mixture. The observed activities of the mixtures as percentages of the predicted values were: soluble starch synthase, 69 ± 10%; branching enzyme, 86 ± 3%; sucrose synthase, 113 ± 8% (means ± S.E.M., $n = 3$).

Our estimates of the maximum catalytic activities of enzymes related to starch synthesis in GUS-control and six different *glgC-16*-transformed lines are given in Table 2. The activity of ADPglucose pyrophosphorylase in each of the *glgC*-16 lines was confirmed as being significantly higher ($P < 0.05$) than that in the GUS-control tubers. For starch synthase, analysis of variance shows that the activity in *glgC-16* line 139 was significantly greater ($P < 0.05$) than that in the GUS-control. However, if the values for starch synthase from all six *glgC-16* lines are treated as a single population and compared with the GUS-control lines, then no significant difference is found. For each of the other enzymes, no statistically significant difference was found between any one *glgC-16* line and the GUS-control.

DISCUSSION

We argue that our estimates of enzyme activity are adequately authenticated and reflect the maximum catalytic activities in the tubers. Thus we conclude that the transformation procedure was effective in that it produced a range of tubers with increased activity of ADPglucose pyrophosphorylase. That this increase was due to the presence of the glgC-16 protein is shown by our Western blot analysis and the demonstration of a linear relationship between enzyme activity and amount of protein (Figures 2 and 3).

Our cell fractionation studies failed to reveal any difference in the distribution of ADPglucose pyrophosphorylase between GUS-control and *glgC-16* transgenic tubers. The close correlation between the distribution of ADPglucose pyrophosphorylase and inorganic pyrophosphatase, the plastid marker, in GUS-control and *glgC-16* tuber extracts strongly suggests that ADPglucose pyrophosphorylase is located in the plastids in both types of tuber.

The *glgC-16*-transformed lines did not show any pleiotropic effect of the increased activity of ADPglucose pyrophosphorylase. We conclude that the *glgC-16*-transformed tubers described in this paper show enhanced activity of ADPglucose pyrophosphorylase due to the presence of the glgC-16 protein. We also suggest that the glgC-16 protein is correctly located in the cell and that no serious pleiotropic changes have occurred. Thus the *glgC-16* tubers may be used to analyse the role of the pyrophosphorylase in starch synthesis, and this is investigated further in the accompanying paper [30].

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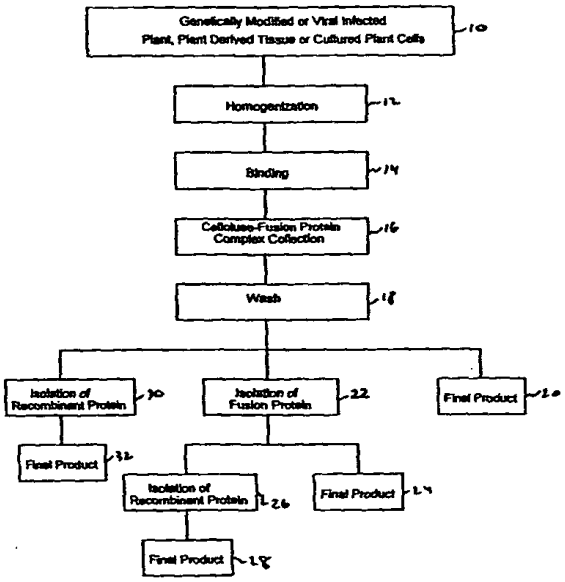
(54) Title: PROCESS OF EXPRESSING AND ISOLATING RECOMBINANT PROTEINS AND RECOMBINANT PROTEIN PRODUCTS FROM PLANTS, PLANT DERIVED TISSUES OR CULTURED PLANT CELLS



(57) Abstract: A process of expressing a recombinant protein in a plant and of isolating the recombinant protein from the plant, the process is effected by (a) providing a plant, a plant derived tissue or cultured plant cells expressing a fusion protein including the recombinant protein and a cellulose binding peptide being fused thereto, the fusion protein being compartmentalized within cells of the plant, plant derived tissue or cultured plant cells, so as to be sequestered from cell walls of the cells of the plant, plant derived tissue or

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cultured plant cells; (b) homogenizing the plant, plant derived tissue or cultured plant cells, so as to bring into contact the fusion protein with a cellulosic matter of the plant, plant derived tissue or cultured plant cells, to thereby effect affinity binding of the fusion protein via the cellulose binding peptide to the cellulosic matter, thereby obtaining a fusion protein cellulosic matter complex; and (c) isolating the fusion protein cellulosic matter complex.

PROCESS OF EXPRESSING AND ISOLATING RECOMBINANT PROTEINS AND RECOMBINANT PROTEIN PRODUCTS FROM PLANTS, PLANT DERIVED TISSUES OR CULTURED PLANT CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a process of expressing and isolating recombinant proteins and recombinant protein products from plants, plant derived tissue or cultured plant cells, which process exploits (i) the high affinity between cellulose binding peptides and cellulose; (ii) the inherent abundance of cellulose in *planta*; and/or (iii) the simplicity associated with cellulose isolation from plants, plant derived tissue and cultured plant cells.

More particularly, the present invention relates to a process expressing and isolating recombinant proteins and recombinant protein products from plants, plant derived tissue or cultured plant cells, which process employs the expression of a fusion protein including a recombinant protein and a cellulose binding peptide fused thereto, plant homogenization, isolation of a fusion protein cellulosic matter complex and optional subsequent isolation of the fusion protein and/or the recombinant protein from the complex. The present invention further relates to nucleic acid molecules and to genetically modified or viral infected plants or plant cells which are useful while implementing the process, and further to a novel composition of matter which results from the process.

Citation or identification of any reference in this section or in any other section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

With the advent of recombinant technology, techniques for the genetic transformation of various host organisms, such as bacteria, yeasts, fungi, plants and animals, for the purposes of producing specific proteins through the expression of heterologous or foreign genes have been extensively developed.

Using these recombinant techniques and hosts, numerous commercially important recombinant proteins (examples of which are included hereinbelow) have been expressed and purified. Expression and isolation of a protein of interest on a commercial scale, neccesitate the selection of a suitable expression host. This selection largely depends on the economics of production and purification, as well as the ability of the host to accomplish the post-translational modifications needed for full biological activity of the recombinant protein.

Much of the early work in biotechnology was directed toward the expression of recombinant or "heterologous" proteins in prokaryotes like *Escherichia coli* and *Bacillus subtilis* because of the relative ease of genetic manipulation, growth in batch culture and large-scale fermentation of prokaryotes.

Although *E. coli* can in certain cases perform some post translational modifications and events, such as, protein folding and disulfide bond formation, it cannot secrete proteins extracellularly nor can it glycosylate, gamma carboxylate, beta hydroxylate, acetylate or process pre- and pro-peptides. *B. subtilis* suffers from the same limitations as *E. coli* except that it is capable of extracellular secretion.

Furthermore, *E. coli* and other bacteria are pathogens and therefore, depending on the application, contaminants such as pyrogens and endotoxins expressed along with the recombinant protein must be removed. In addition, extensive post-purification chemical and enzymatic treatments (e.g., to refold the protein into an active form) are sometimes required in order to obtain a biologically active protein.

Because proteins are not secreted from prokaryotes like *E. coli*, bacterial cells must be disrupted for product recovery. The subsequent release of bacterial contaminants and other proteins make product purification more difficult and expensive. Because purification accounts for up to 90 % of the total cost of producing recombinant proteins in bacteria, proteins like Insulin can cost several thousand dollars per gram when recombinantly produced in, and subsequently isolated from, *E. coli*.

Because of the many limitations associated with prokaryotic hosts, the biotechnology industry has looked for eukaryotic host cultures such as, yeast, fungi, insect cells, and mammalian cell tissue culture, to properly and efficiently express recombinant proteins.

For most of the proteins requiring extensive post-translational modifications for therapeutic and/or functional activity, mammalian cell culture is the most common alternative to *E. coli*. Although mammalian cells are capable of correctly folding and glycosylating bioactive proteins, the quality and extent of glycosylation can vary with different culture conditions among the same host cells. Furthermore, mammalian culture has extremely high fermentation costs (60-80% of total production expense), requires expensive media, and poses safety concerns from potential contamination by viruses and other pathogens. Yields are generally low and

in the range of 0.5-1.5% of cellular protein, or micrograms per liter (up to 300-400 milligrams per liter).

Yeast, other fungi, and insect cells are currently being used as alternatives to mammalian cell culture. Yeast, however, produces incorrectly glycosylated proteins that have excessive mannose residues and are generally limited in eukaryotic processing. Further, although the baculovirus insect cell system can produce high levels of glycosylated proteins, these are typically not secreted, making purification complex and expensive. Fungi represent the best current system for high-volume, low-cost production of recombinant proteins, but they are not capable of expressing many target proteins.

In addition, eukaryotic cultures, require the maintenance of suitable conditions for efficient commercially viable expression of proteins. As such, the ambient temperature, pH value and aeration level of such cultures need to be carefully controlled, while nutrients must be added to the culture medium in carefully regulated doses and waste products removed. In addition, rigorous aseptic practices must be observed in order to avoid contamination by extraneous microbes. Such cultures are thus normally grown in sophisticated fermentors or bioreactors which are housed in expensively maintained factories. Such overheads are reflected in the high price of the recombinant protein end-products.

To a lesser extent, animals have also been utilized for the production of recombinant proteins. Although large amounts of protein can be produced and relatively easily recovered from such animals (e.g., proteins specifically produced in mammary glands and secreted with the milk), production in such host is limited to the expression of proteins which do not interfere with the host physiology. In addition, transgenic animals are subject to lengthy lead times to develop herds with stable genetics, high operating costs, contamination by animal viruses and a relatively slow rate of biomass generation substantially prolonging the time period following which recovery of commercial amounts of the protein can be effected.

The biochemical, technical and economic limitations on existing prokaryotic and eukaryotic expression systems has created substantial interest in developing new expression systems for the production of recombinant proteins.

Plants represent the most likely alternative to existing expression systems. With the availability and on going development of plant

transformation techniques, most commercially important plant species can now be genetically modified to express a variety of recombinant proteins.

Such transformation techniques include, for example, the *Agrobacterium* vector system, which involves infection of the plant tissue with a bacterium (*Agrobacterium*) into which the foreign gene has been inserted. A number of methods for transforming plant cells with *Agrobacterium* are well known (Klee *et al.*, Annu. Rev. Plant Physiol. (1987) 38:467-486; Schell and Vasil Academic Publishers, San Diego, Calif. (1989) p. 2-25; and Gatenby (1989) in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. p. 93-112).

The biolistic or particle gun method, which permits genetic material to be delivered directly into intact cells or tissues by bombarding regeneratable tissues, such as meristems or embryogenic callus, with DNA-coated microparticles has contributed to plant transformation simplicity and efficiency. The microparticles penetrate the plant cells and act as inert carriers of a genetic material to be introduced therein. Microprojectile bombardment of embryogenic suspension cultures has proven successful for the production of transgenic plants of a variety of species. Various parameters that influence DNA delivery by particle bombardment have been defined (Klein *et al.*, Bio/Technology (1998) 6:559-563; McCabe *et al.*, Bio/Technology (1998) 6:923-926; and Sanford, Physiol. Plant. (1990) 79:206-209).

Micropipette systems are also used for the delivery of foreign DNA into plants via microinjection (Neuhaus *et al.*, Theor. Appl. Genet. (1987) 75:30-36; and Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217).

Other techniques developed to introduce foreign genes into plants include direct DNA uptake by plant tissue, or plant cell protoplasts (Schell and Vasil (1987) Academic Publishers, San Diego, Calif. p. 52-68; and Toriyama *et al.*, Bio/Technology (1988) 6:1072-1074) or by germinating pollen (Chapman, Mantell and Daniels (1985) W. Longman, London, p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719).

DNA uptake induced by brief electric shock of plant cells has also been described (Zhang *et al.*, Plant. Cell. Rep. (1988) 7:379-384 and Fromm *et al.*, Nature (1986) 319:791-793).

In addition, virus mediated plant transformation has also been extensively described. Transformation of plants using plant viruses is

described, for example, in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693, EPA 194,809, EPA 278,667, and Gluzman *et al.*, (1988) Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189. Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, have also been described , for reference, see, for example WO 87/06261.

The production of recombinant proteins and peptides in plants has been investigated using a variety of approaches including transcriptional fusions using a strong constitutive plant promoter (e.g., from cauliflower mosaic virus, Sijmons *et al.*, Bio/Technology (1990) 8:217-221); transcriptional fusions with organ specific promoter sequences (Radke *et al.*, Theoret. Appl. Genet. (1988) 75:685-694); and translational fusions which require subsequent cleavage of a recombinant protein (Vanderkerckove *et al.*, Bio/Technology (1989) 7:929-932).

The application of such genetic transformation techniques has allowed the incorporation of a variety of important genetic traits for crop improvement and also for the biotechnological production of extractable, valuable, foreign proteins including enzymes, vaccine proteins and antibodies.

Foreign proteins that have been successfully expressed in plant cells include proteins from bacteria (Fraley *et al.* Proc. Natl. Acad. Sci. U.S.A (1993) 80:4803-4807), animals (Misra and Gedamu, Theor. Appl. Genet. (1989) 78:161-168), fungi and other plant species (Fraley *et al.* Proc. Natl. Acad. Sci. U.S.A. (1983) 80:4803-4807). Some proteins, predominantly markers of DNA integration, have been expressed in specific cells and tissues including seeds (Sen Gupta-Gopalan *et al.* Proc. Natl. Acad. Sci. U.S.A. (1985) 82:3320-3324; Radke *et al.* Theor. Appl. Genet. (1988) 75:685-694).

Due to the advantageous economics of field-grown crops, the ability to synthesize proteins in storage organs like tubers, seeds, fruits and leaves and the ability of plants to perform many of the post-translational modifications previously described, several plant expression systems are currently investigated for potential as highly effective and economically feasible systems for the production of recombinant proteins.

Since highly expressive systems such as the ubiquitin fusion system described in U.S. Pat. No. 5,773,705 have been demonstrated, a major hurdle to an effective plant expression system resides with the relatively

complicated purification procedures necessary in order to purify the recombinant protein.

As such, alternative expression approaches have been undertaken in an effort to simplify the purification procedure of the recombinant protein from the plant cells.

One such system focuses on the use of seed-storage protein promoters as a means of deriving seed-specific expression. Using such a system, Vanderkerckove *et al.*, (Bio/Technol. (1989) 7:929-932) expressed the peptide Leu-enkephalin in seeds of *Arabidopsis thaliana* and *Brassica napus*. The level of expression of this peptide was quite low and it appeared that expression of this peptide was limited to endosperm tissue.

Another system utilizing seeds as an expression host is disclosed in U.S. Pat. No. 5,888,789. This system provides for the secretion of heterologous protein by malting of monocot plant seeds. The heterologous genes are expressed during germination of the seeds and isolated from a malt.

U.S. Pat. No. 5,580,768 describes a method of producing a genetically transformed fluid-producing plant. The genetically transformed plant which can be for example, a rubber secreting (*Hevea*) plant is capable of expressing the target product in the fluid that it produces which in this case is latex.

U.S. Pat No. 5,650,554 describes the use of a class of genes called oil body protein genes, that have unique features, allowing the production of recombinant proteins that can be easily separated from other host cell components.

Many additional expression systems have been described utilizing specific targeting or directing of recombinant proteins to specific plant tissues.

Although systems which target or direct recombinant protein production to specific tissues allow for easier recombinant protein isolation such systems are typically limited in the effective host range and/or the amounts of recombinant proteins produced since such systems fail to exploit the entire plant biomass.

A novel approach for simplifying the purification of recombinant enzymes from plant host cells is disclosed in U.S. Pat. No. 5,474,925 which describes an expression construct utilizing a signal peptide translationally fused to a recombinant enzyme which targets the enzyme to the cellulose matrix of the cell wall. This enables the isolation of the

enzyme along with the easily recoverable cellulose matrix. This system is utilized for the localized expression of commercially important enzymes in cotton fibers. According to this system, the expressed enzymes are recovered along with the cellulosic matter of the fibers. The enzyme-cellulose matrix recovered, is directly utilized for commercial enzymatic processes.

Although this system presents a simple means with which a recombinant protein can be expressed and isolated, it is limited to the production of enzymes in cotton fibers of the cotton plant.

Furthermore, a major hurdle encountered when expressing cellulose targeted proteins within a plant is the interference of the expressed products in the natural formation of the cell wall, which typically results in growth arrest of the plant growth. Although this hurdle can be overcome by, for example, targeting the protein to specific plant tissue as is the case for U.S. Pat. No. 5,474,925, this targeting severely limits the expressing biomass and as such the quantity of the expressed protein. In addition, targeting the expression to a specific plant tissue also limits the number of plant species which can be effectively utilized for such an expression.

There is thus a widely recognized need for, and it would be highly advantageous to have, a plant expression system and method which provide high level of expression of a recombinant protein and which allow simple and effective recovery of the expressed recombinant protein devoid of the above limitations.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a process of expressing a recombinant protein in a plant and of isolating the recombinant protein from the plant, the process comprising the steps of (a) providing a plant, a plant derived tissue or cultured plant cells expressing a fusion protein including the recombinant protein and a cellulose binding peptide being fused thereto, the fusion protein being compartmentalized within cells of the plant, plant derived tissue or cultured plant cells, so as to be sequestered from cell walls of the cells of the plant, plant derived tissue or cultured plant cells; (b) homogenizing the plant, plant derived tissue or cultured plant cells, so as to bring into contact the fusion protein with a cellulosic matter of the plant, plant derived tissue or cultured plant cells, to thereby effect affinity binding of the fusion protein via the cellulose binding peptide to the cellulosic matter, thereby obtaining a fusion protein cellulosic

matter complex; and (c) isolating the fusion protein cellulosic matter complex.

According to further features in preferred embodiments of the invention described below, the process further comprising the steps of washing the fusion protein cellulosic matter complex, thereby removing endogenous plant proteins and other plant material therefrom and collecting the fusion protein cellulosic matter complex as a final product of the process.

According to still further features in the described preferred embodiments the process further comprising the steps of washing the fusion protein cellulosic matter complex, thereby removing endogenous plant proteins and other plant material therefrom, exposing the fusion protein cellulosic matter complex to conditions effective in dissociating the fusion protein from the cellulosic matter; and isolating the fusion protein, thereby obtaining an isolated fusion protein.

According to still further features in the described preferred embodiments the process further comprising the steps of exposing the isolated fusion protein to conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom, thereby obtaining a released recombinant protein and isolating the released recombinant protein.

According to still further features in the described preferred embodiments the process further comprising the steps of washing the fusion protein cellulosic matter complex, thereby removing endogenous plant proteins and other plant material therefrom, exposing the fusion protein cellulosic matter complex to conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom, thereby obtaining a released recombinant protein, and isolating the released recombinant protein.

According to still further features in the described preferred embodiments, the conditions effective in dissociating the fusion protein from the cellulosic matter are selected from the group consisting of basic conditions, denaturative conditions and affinity displacement conditions.

According to still further features in the described preferred embodiments, the conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom are selected from the group consisting of proteolysis effected via a protease and proteolysis effected under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

According to another aspect of the present invention there is provided a genetically modified or viral infected plant or cultured plant cells expressing a fusion protein including a recombinant protein and a cellulose binding peptide

According to further features in preferred embodiments of the invention described below, the fusion protein is compartmentalized within cells of the plant or cultured plant cells, so as to be sequestered from cell walls of the cells of the plant or cultured plant cells.

According to still further features in the described preferred embodiments the fusion protein is compartmentalized within a cellular compartment selected from the group consisting of cytoplasm, endoplasmic reticulum, golgi apparatus, oil bodies, starch bodies, chloroplastids, chloroplasts, chromoplastids, chromoplasts, vacuole, lysosomes, mitochondria, and nucleus.

According to still further features in the described preferred embodiments expression of the fusion protein is under a control of a constitutive or tissue specific plant promoter.

According to still further features in the described preferred embodiments the fusion protein includes a recombinant protein and a cellulose binding peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

According to yet another aspect of the present invention there is provided a composition of matter comprising (a) a plant derived cellulosic matter; and (b) a fusion protein including a recombinant protein and a cellulose binding peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, the fusion protein being complexed to the plant derived cellulosic matter by affinity binding via the cellulose binding peptide.

According to still another aspect of the present invention there is provided a nucleic acid molecule comprising (a) a promoter sequence for directing protein expression in plant cells; (b) a heterologous nucleic acid sequence including (i) a first sequence encoding a cellulose binding peptide; (ii) a second sequence encoding a recombinant protein, wherein the first and second sequences are joined together in frame; optionally (iii) a third sequence encoding a signal peptide for directing a protein to a cellular

compartment, the third sequence being upstream and in frame with the first and second sequences; and/or optionally (iv) a fourth sequence encoding a unique amino acid sequence being recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, the fourth sequence being between and in frame with the first and second sequences, wherein, the heterologous nucleic acid sequence being down stream the promoter sequence, such that expression of the heterologous nucleic acid sequence is effectable by the promoter sequence.

According to further features in preferred embodiments of the invention described below, the nucleic acid molecule further comprising a sequence element selected from the group consisting of an origin of replication for propagation in bacterial cells, at least one sequence element for integration into a plant's genome, a polyadenylation recognition sequence, a transcription termination signal, a sequence encoding a translation start site, a sequence encoding a translation stop site, plant RNA virus derived sequences, plant DNA virus derived sequences, tumor inducing (Ti) plasmid derived sequences, and a transposable element derived sequence.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a novel process of expressing and isolating recombinant proteins and recombinant protein products from plants, plant derived tissue or cultured plant cells, which process exploits (i) the high affinity between cellulose binding peptides and cellulose; (ii) the inherent abundance of cellulose in *planta*; and (iii) the simplicity associated with cellulose isolation from plants, plant derived tissue and/or cultured plant cells.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the

invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a process flow chart demonstrating the process according to the present invention.

FIGs. 2a-b are nucleotide (2a) and restriction maps (2b) of the insert into the pUC19-cel1-ProtL-cexNG vector constructed in accordance with the teachings of the present invention.

FIGs. 3a-b are nucleotide (3a) and restriction maps (3b) of the insert into the pBS-Sig-cex-Fx-HDEL vector constructed in accordance with the teachings of the present invention.

FIGs. 4a-b are nucleotide (4a) and restriction maps (4b) of the insert into the pBS-Sig-Tma-Fx-HDEL vector constructed in accordance with the teachings of the present invention.

FIG. 5 is a flow chart diagram depicting the step involved in the analysis of the transgenic plant material produced according to the teachings of the present invention.

FIG. 6 is a gel image of PCR amplified fragments from ProtL-cex transformants 1-15. N - negative control, M - molecular weight marker (MWM).

FIGs. 7a-b are gel images of PCR amplified fragments from cex-fx transformants 1-19. N - negative control, M - molecular weight marker (MWM).

FIGs. 8a-b are gel images of PCR amplified fragments from Tma-fx transformants 1-19. C - positive control, M - molecular weight marker (MWM).

FIGs. 9a-b are immunoblot images of proteins extracted from ProtL-cexNG transformants. The extracted proteins were separated on SDS-PAGE, blotted, and reacted with anti CBDcex antibody(6a) or mouse IgG (6b) as is further described in Example 2. M - MWM, a -a WT plant cell wall fraction, b - transformant line 2 cell wall fraction, c - WT cellulose fraction (exogenous), d - transformant line 2 cellulose fraction.

FIGs. 10a-b are immunoblot images of proteins extracted from CBDcex-fx transformants. The extracted proteins were separated on SDS-PAGE, blotted, and reacted with anti-Fx (7a) or anti-CBDcex (7b) antibodies as is further described in Example 2. C - positive control, M - MWM, wt - WT plant line, 5-24 - transformant plant lines.

FIGs. 11a-d are immunoblot images of proteins extracted from CBDTma-fx transformants. The extracted proteins were separated on SDS-PAGE, blotted, and reacted with anti-Fx antibodies as is further described in Example 2. Figures 11a and 11c represent proteins extracted from the cellulose fraction, while Figures 11b and 11d represent proteins extracted from the wall fraction. C - positive control, M - MWM, wt - WT plant line, 2-19 - transformant plant lines.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a process which can be used for expressing and isolating recombinant proteins and recombinant protein products from plants, plant derived tissue or cultured plant cells. The present invention is further of nucleic acid molecules and genetically modified or viral infected plants or plant cells which are useful while implementing the process, and further of a novel composition of matter which results from the process. Specifically, the present invention can be used to obtain large quantities of the recombinant proteins and the recombinant protein products in a simple and cost effective manner, since the process according to the present invention exploits (i) the high affinity between cellulose binding peptides and cellulose; (ii) the inherent abundance of cellulose in *planta*; and (iii) the simplicity associated with cellulose isolation from plants, plant derived tissue and/or cultured plant cells.

The principles and operation of a process according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Process:

Referring now to the drawings, Figure 1 is a flow chart illustrating the process according to the teachings of the present invention.

The process according to the present invention is directed at expressing a recombinant protein in a plant and further at isolating the recombinant protein from the plant. The process according to the present invention is effected by first providing a plant, a plant derived tissue or cultured plant cells (which are referred to herein below individually and collectively as "plant material") **10** expressing a fusion protein which includes the recombinant protein and a cellulose binding peptide fused thereto. The fusion protein is compartmentalized within cells of the plant material, so as to be sequestered from cell walls of the cells of the plant material. As used herein in the specification and in the claims section that follows, the phrase "cultured plant cells" includes both non-differentiated plant cell cultures and some what more differentiated callus cultures.

Compartmentalizing and thereby sequestering the fusion protein from the cell walls is an essential feature of the present invention because high levels of exspressed cellulose binding peptide associated with plant cell walls inhibit plant growth to a great extent. See to this effect U.S. Pat. applications Nos. 09/006,632; 09/006,636; and PCT/IL98/00345 (WO 99/07830).

When sufficient expression has been detected by sampling and testing the plant material as further detailed hereinunder, the plant material is homogenized **12** so as to bring into contact the fusion protein with a cellulosic matter of the plant material, to thereby effect affinity binding **14** of the fusion protein via the cellulose binding peptide to the cellulosic matter, thereby obtaining a fusion protein cellulosic matter complex. Conditions such as, but not limited to, temperature, pH, salt concentration, time and the like are preferably set so as to allow maximal binding. Such conditions are well know to the skilled artisan and can be experimentally modified to best suit a specific application. Sampling and testing can be employed to monitor the binding process, as further detailed hereinunder.

When sufficient binding has occurred the fusion protein cellulosic matter complex is collected or isolated **16** by methods well known to the skilled artisan which method are traditionally employed for isolation of cellulosic matter from plant material. Thereafter, a wash step **18** is employed to remove unbound material, including, in particular, unbound endogenous plant proteins, thereby isolating the fusion protein cellulosic matter complex. The wash step can be repeated one or several times with a single or several wash solutions, each of which can include in addition to water, buffers, salts, detergents and the like to efficiently effect the removal

of unbound matter from the fusion protein cellulosic matter complex. The wash step can be effected in solution using appropriate stiring, however, advantageously, the wash step is effected within a column into which the collected or isolated fusion protein cellulosic matter complex is packed and subsequently washed.

According to one embodiment of the present invention, and as indicated in Figure 1 by numeral **20**, the fusion protein cellulosic matter complex is collected as a final product of the process. Such a final product can serve as a pack for affinity columns. In this case the recombinant protein is selected to have affinity to a ligand, which can then be affinity purified via a column packed with the fusion protein cellulosic matter complex, in a manner otherwise similar to that described in U.S. Pat. No. 5,474,925, which is incorporated herein by reference. One of the advantages of the process described herein over the teachings of U.S. Pat. No. 5,474,925 is that by sequestering the fusion protein from the cell walls one can achieve very high expression of the fusion protein as compared to the low expression levels practically enabled by U.S. Pat. No. 5,474,925, because no deleterious effect on plant growth is exerted. As a result, the specific activity of the fusion protein cellulosic matter complex formed according to the present invention, i.e., the number of fusion protein molecules per weight of cellulosic matter, is far superior. Further details relating to the effect of high cellulose binding peptide expression on plant development see also PCT/IL98/000345.

According to another embodiment of the present invention, as indicated by numeral **22**, the final product of the process according to the present invention is the fusion protein itself **24**. Thus, according to this embodiment of the present invention, conditions effective in dissociating the fusion protein from the cellulosic matter are used to effect such dissociation. The dissociated fusion protein is thereafter readily isolated by any conventional separation technique, such as, but not limited to, elution or size separation, such as differential filtration or centrifugation, thereby obtaining an isolated fusion protein. Conditions effective in dissociating the fusion protein from the cellulosic matter include, but are not limited to, basic conditions (e.g., 20 mM Tris pH 12) which are known to dissociate all cellulose binding peptides from cellulose, denaturative conditions, or affinity displacement conditions, e.g., using 200 nM glucose or cellobiose which are know in their ability to elute family IX cellulose binding domains (CBDs). Alternatively, a protein cleavage site can be inserted in the

cellulose binding peptide to facilitate the dissociation of the fusion protein by specific proteolysis, for example. See to this effect and to other uses of CBD-fusion proteins U.S. Pat. Nos. 5,719,044; 5,670,623; 5,856,021; 5,137,819; 5,202,247; 5,340,731; and 5,474,925; and U.S. Pat. applications Nos. 08/788,621; and 08/788,622; EP 0 381 719 B1, and EP application No. 93907724.4. See also the teachings of U.S. Pat. No. 5,834,247, which is further described hereinunder.

As indicated by numeral **26**, the fusion protein thus isolated can be exposed to conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom, thereby obtaining a released recombinant protein which can be thereafter isolated as a final product **28**. Conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom include, but are not limited to, proteolysis effected via a protease, such as, but not limited to, Factor Xa, enterokinase, thrombin, trypsin, papain, pepsin, chemotrypsin and the like, or proteolysis effected via controllable intervening protein sequence (CIVPS) inserted into or adjacent the cellulose binding peptide, the CIVPS are capable of excision from or cleavage of the peptide under predetermined conditions in cis or in trans, e.g., increase in temperature, exposure to light, unblocking of amino acid residues by dephosphorylation and treatment with chemical reagents or deglycosylation, examples include proteolysis effected under acidic conditions (HCl, e.g., to cleave between Asp and Pro) and proteolysis effected by a proteolysing reagent, such as CNBr to cleave downstream of Met, all as known in the art and/or as further described in U.S. Pat. No. 5,834,247, which is incorporated herein by reference.

Isolating final product **28** from other proteolytic products derived, for example, from the cellulose binding peptide, can be effected by any one of a number of protein isolation techniques well known to the skilled artisan, including, but not limited to, affinity separation via, for example, antibodies bound to a solid support, size and/or charge based separation via gel electrophoresis or chromatography, and the like. Additional methods include, but are not limited to, fractionation, gel-filtration, ion-exchange, hydrophobic, and affinity chromatography, ultrafiltration and crystallization.

According to an alternative embodiment of the process of the present invention, as indicated in Figure 1 by numeral **30**, the washed fusion protein cellulose matter complex resulting from step **18** is exposed to conditions effective in digesting the fusion protein so as to release the recombinant protein therefrom, thereby obtaining a released recombinant protein. These

conditions are similar to those described with respect to step **26**. The released recombinant protein is thereafter readily isolated by any conventional separation technique, such as, but not limited to, displacement or size separation, such as differential filtration or centrifugation, thereby obtaining an isolated recombinant protein final product **32**.

Cellulose binding peptides:

As used herein in the specification and in the claims section below, the phrase "cellulose binding peptide" includes peptides e.g., proteins and domains (portions) thereof, which are capable of, when expressed in plant cells, affinity binding to a plant derived cellulosic matter following homogenization and cell rupture. The phrase thus includes, for example, peptides which were screened for their cellulose binding activity out of a library, such as a peptide library or a DNA library (e.g., a cDNA expression library or a display library) and the genes encoding such peptides isolated and are expressible in plants. Yet, the phrase also includes peptides designed and engineered to be capable of binding to cellulose and/or units thereof.

Such peptides include amino acid sequences expressible in plants that are originally derived from a cellulose binding region of, e.g., a cellulose binding protein (CBP) or a cellulose binding domain (CBD). The cellulose binding peptide according to the present invention can include any amino acid sequence expressible in plants which binds to a cellulose polymer. For example, the cellulose binding domain or protein can be derived from a cellulase, a binding domain of a cellulose binding protein or a protein screened for, and isolated from, a peptide library, or a protein designed and engineered to be capable of binding to cellulose or to saccharide units thereof, and which is expressible in plants. The cellulose binding domain or protein can be naturally occurring or synthetic, as long as it is expressible in plants. Suitable polysaccharidases from which a cellulose binding domain or protein expressible in plants may be obtained include β-1,4-glucanases. In a preferred embodiment, a cellulose binding domain or protein from a cellulase or scaffoldin is used. Typically, the amino acid sequence of the cellulose binding peptide expressed in plants according to the present invention is essentially lacking in the hydrolytic activity of a polysaccharidase (e.g., cellulase, chitinase), but retains the cellulose binding activity. The amino acid sequence preferably has less than about 10 % of the hydrolytic activity of the native polysaccharidase; more preferably less than about 5 %, and most preferably less than about 1 % of

the hydrolytic activity of the native polysaccharidase, ideally no activity altogether.

The cellulose binding domain or protein can be obtained from a variety of sources, including enzymes and other proteins which bind to cellulose which find use in the subject invention.

In Table 4 below are listed those binding domains which bind to one or more soluble/insoluble polysaccharides including all binding domains with affinity for soluble glucans (α, β, and/or mixed linkages). The N1 cellulose-binding domain from endoglucanase CenC of *C. fimi* is the only protein known to bind soluble cellosaccharides and one of a small set of proteins which are known to bind any soluble polysaccharides. Also, listed in Tables 1 to 3 are examples of proteins containing putative β-1,3-glucan-binding domains (Table 1); proteins containing Streptococcal glucan-binding repeats (Cpl superfamily) (Table 2); and enzymes with chitin-binding domains, which may also bind cellulose (Table 3). The genes encoding each one of the peptides listed in Tables 1-4 are either isolated or can be isolated as further detailed hereinunder, and therefore, such peptides are expressible in plants. Scaffoldin proteins or portions thereof, which include a cellulose binding domain, such as that produced by *Clostridium cellulovorans* (Shoseyov *et al.*, PCT/US94/04132) can also be used as the cellulose binding peptide expressible in plants according to the present invention. Several fungi, including *Trichoderma* species and others, also produce polysaccharidases from which polysaccharide binding domains or proteins expressible in plants can be isolated. Additional examples can be found in, for example, Microbial Hydrolysis of Polysaccharides, R. A. J. Warren, Annu. Rev. Microbiol. 1996, 50:183-212; and "Advances in Microbial Physiology" R. K. Poole, Ed., 1995, Academic Press Limited, both are incorporated by reference as if fully set forth herein.

Table 1

Overview of proteins containing putative β-1,3 glucan-binding domains

Source (strain)	Protein	accession No.	Ref[1]
Type I			
B. circulans (WL-12)	GLCA1	P23903/M34503/JQ0420	1
B. circulans (IAM 1165)	BglH	JN0772/D17519/S67033	2

Type II

Actinomadura sp. (FC7)	XynII	U08894	3
Arthrobacter sp. (YCWD3)	GLCI	D23668	9
O. xanthineolytica	GLC	P22222/M60826/A39094	4
R. faecitabidus (YLM-50)	RP I	Q05308/A45053/D10753	5a,b
R. communis	Ricin	A12892	6
S. lividans (1326)	XlnA	P26514/M64551/JS07986	7
T. tridentatus	FactorGa	D16622	8

B. : *Bacillus*, *O.* : *Oerskovia*, *R. faecitabidus* : *Rarobacter faecitabidus*, *R. communis*: *Ricinus communis*, *S.* : *Streptomyces*, *T.* : *Tachypleus* (Horseshoe Crab)

[1]**References:**

1) Yahata *et al.* (1990) *Gene* **86**, 113-117
2) Yamamoto *et al.* (1993) *Biosci. Biotechnol. Biochem.* **57**, 1518-1525
3) Harpin *et al.* (1994) *EMBL Data Library*
4) Shen *et al.* (1991) *J. Biol. Chem.* **266**, 1058-1063
5a) Shimoi *et al.* (1992) *J. Biol. Chem.* **267**, 25189-25195
5b) Shimoi *et al.* (1992) *J. Biochem* **110**, 608-613
6) Horn *et al.* (1989) Patent A12892
7) Shareck *et al.* (1991) *Gene* **107**, 75-82
8) Seki *et al.* (1994) *J. Biol. Chem.* **269**, 1370-1374
9) Watanabe *et al.* (1993) *EMBL Data Library*

Table 2

Overview of proteins containing Streptococcal glucan-binding repeats (Cpl superfamily)

Source	Protein	Accession No.	Ref.[2]
S. downei (sobrinus) (0MZ176)	GTF-I	D13858	1
S. downei (sobrinus) (MFe28)	GTF-I	P11001/M17391	2
S. downei (sobrinus) (MFe28)	GTF-S	P29336/M30943/A41483	3
S. downei (sobrinus) (6715)	GTF-I	P27470/D90216/A38175	4
S. downei (sobrinus)	DEI	L34406	5
S. mutants (Ingbritt)	GBP	M30945/A37184	6
S. mutants (GS-5)	GTF-B	A33128	7
S. mutants (GS-5)	GTF-B	P08987/M17361/B33135	8
S. mutants	GTF-B$^{3'\text{-ORF}}$	P05427/C33135	8
S. mutants (GS-5)	GTF-C	P13470/M17361/M22054	9
S. mutants (GS-5)	GTF-C	not available	10
S. mutants (GS-5)	GTF-D	M29296/A45866	11
S. salivarius	GTF-J	A44811/S22726/S28809 Z11873/M64111	12
S. salivarius	GTF-K	S22737/S22727/Z11872	13
S. salivarius (ATCC25975)	GTF-L	L35495	14
S. salivarius (ATCC25975)	GTF-M	L35928	14
S. pneumoniae R6	LytA	P06653/A25634/M13812	15
S. pneumoniae	PspA	A41971/M74122	16

Phage HB-3	HBL	P32762/M34652	17
Phage Cp-1	CPL-1	P15057/J03586/A31086	18
Phage Cp-9	CPL-9	P19386/M34780/JQ0438	19
Phage EJ-1	EJL	A42936	20
C. difficile (VPI 10463)	ToxA	P16154/A37052/M30307 X51797/S08638	21
C. difficile (BARTS W1)	ToxA	A60991/X17194	22
C. difficile (VPI 10463)	ToxB	P18177/X53138/X60984 S10317	23,24
C. difficile (1470)	ToxB	S44271/Z23277	25,26
C. novyi	a-toxin	S44272/Z23280	27
C. novyi	a-toxin	Z48636	28
C. acetobutylicum (NCIB8052)	CspA	S49255/Z37723	29
C. acetobutylicum (NCIB8052)	CspB	Z50008	30
C. acetobutylicum (NCIB8052)	CspC	Z50033	30
C. acetobutylicum (NCIB8052)	CspD	Z50009	30

[2]References:

1) Sato *et al.* (1993) *DNA sequence* **4**, 19-27
2) Ferreti *et al.* (1987) *J. Bacteriol.* **169**, 4271-4278
3) Gilmore *et al.* (1990) *J. Infect. Immun.* **58**, 2452-2458
4) Abo *et al.* (1991) *J. Bacteriol.* **173**, 989-996
5) Sun *et al.* (1994) *J. Bacteriol.* **176**, 7213-7222
6) Banas *et al.* (1990) *J. Infect. Immun.* **58**, 667-673
7) Shiroza *et al.* (1990) *Protein Sequence Database*
8) Shiroza *et al.* (1987) *J. Bacteriol.* **169**, 4263-4270
9) Ueda *et al.* (1988) *Gene* **69**, 101-109
10) Russel (1990) *Arch. Oral. Biol.* **35**, 53-58
11) Honda *et al.* (1990) *J. Gen. Microbiol.* **136**, 2099-2105
12) Giffard *et al.* (1991) *J. Gen. Microbiol.* **137**, 2577-2593
13) Jacques (1992) *EMBL Data Library*
14) Simpson *et al.* (1995) *J. Infect. Immun.* **63**, 609-621
15) Gargia *et al.* (1986) *Gene* **43**, 265-272
16) Yother *et al.* (1992) *J. Bacteriol.* **174**, 601-609
17) Romero *et al.* (1990) *J. Bacteriol.* **172**, 5064-5070
18) Garcia *et al.* (1988) *Proc. Natl. Acad. Sci, USA* **85**, 914-918
19) Garcia *et al.* (1990) *Gene* **86**, 81-88
20) Diaz *et al.* (1992) *J. Bacteriol.* **174**, 5516-5525
21) Dove *et al.* (1990) *J. Infect. Immun.* **58**, 480-488
22) Wren *et al.* (1990) *FEMS Microbiol. Lett.* **70**, 1-6
23) Barroso *et a.* (1990) *Nucleic Acids Res.* **18**, 4004-4004
24) von Eichel-Streiber *et al.* (1992) *Mol. Gen. Genet.* **233**, 260-268
25) Sartinger *et al.* (1993) *EMBL Data Library*
26) von Eichel-Streiber *et al.* (1995) *Mol. Microbiol.* **In Press**
27) Hofmann *et al.* (1993) *EMBL Data Library*
28) Hofmann *et al.* (1995) *Mol. Gen. Genet.* **In Press**
29) Sanchez *et al.* (1994) *EMBL Data Library*
30) Sanchez *et al.* (1995) *EMBL Data Library*

New cellulose binding peptides with interesting binding characteristics and specificities can be identified and screened for and the

genes encoding same isolated using well known molecular biology approaches combined with a variety of other procedures including, for example, spectroscopic (titration) methods such as: NMR spectroscopy (Zhu *et al.* Biochemistry (1995) 34:13196-13202, Gehring *et al.* Biochemistry (1991) 30:5524-5531), UV difference spectroscopy (Belshaw *et al.* Eur. J. Biochem. (1993) 211:717-724), fluorescence (titration) spectroscopy (Miller *et al. J. Biol. Chem.* (1983) 258:13665-13672), UV or fluorescence stopped flow analysis (De Boeck *et al.* Eur. J. Biochem. (1985) 149:141-415), affinity methods such as affinity electrophoresis (Mimura *et al.* J. chromatography (1992) 597:345-350) or affinity chromatography on immobilized mono or oligosaccharides, precipitation or agglutination analysis including turbidimetric or nephelometric analysis (Knibbs *et al.* J. Biol. Chem. (1993) 14940-14947), competitive inhibition assays (with or without quantitative IC50 determination) and various physical or physico-chemical methods including differential scanning or isothermal titration calorimetry (Sigurskjold *et al. J. Biol. Chem.* (1992) 267:8371-8376; Sigurskjold *et al.* Eur. J. Biol. (1994) 225:133-141) or comparative protein stability assays (melts) in the absence or presence of oligo saccharides using thermal CD or fluorescence spectroscopy.

The K_a for binding of the cellulose binding domains or proteins to cellulose is at least in the range of weak antibody-antigen extractions, i.e., $\geq 10^3$, preferably 10^4, most preferably 10^6 M^{-1}. If the binding of the cellulose binding domain or protein to cellulose is exothermic or endothermic, then binding will increase or decrease, respectively, at lower temperatures, providing a means for temperature modulation of the binding step, see numeral **14** in Figure 1.

Table 3

Overview of enzymes with chitin-binding domains

Source (strain)	Enzyme	Accession No.	Ref.[3]
Bacterial enzymes			
Type I			
Aeromonas sp. (No10S-24)	Chi	D31818	1
Bacillus circulans (WL-12)	ChiA1	P20533/M57601/A38368	2
Bacillus circulans (WL-12)	ChiD	P27050/D10594	3
Janthinobacterium lividum	Chi69	U07025	4

Streptomyces griseus	Protease C	A53669	5
Type II			
Aeromonas cavia (K1)	Chi	U09139	6
Alteromonas sp (0-7)	Chi85	A40633/P32823/D13762	7
Autographa californica (C6)	NPH-128[a]	P41684/L22858	8
Serratia marcescens	ChiA	A25090/X03657/L01455/P07254	9
Type III			
Rhizopus oligosporus (IFO8631)	Chi1	P29026/A47022/D10157/S27418	10
Rhizopus oligosporus (IFO8631)	Chi2	P29027/B47022/D10158/S27419	10
Saccharomyces cerevisiae	Chi	S50371/U17243	11
Saccharomyces cerevisiae Chi1 (DBY939)		P29028/M74069	12
Saccharomyces cerevisiae Chi2 (DBY918)		P29029/M7407/B41035	12
Plant enzymes			
Hevein superfamily			
Allium sativum	Chi	M94105	13
Amaranthus caudatus	AMP-1[b]	P27275/A40240	14, 15
Amaranthus caudatus	AMP-2[b]	S37381/A40240	14, 15
Arabidopsis thaliana (cv. colombia)	ChiB	P19171/M38240/B45511	16
Arabidopsis thaliana	PHP[c]	U01880	17
Brassica napus	Chi	U21848	18
Brassica napus	Chi2	Q09023/M95835	19
Hevea brasiliensis	Hev1[d]	P02877/M36986/A03770/A38288	20, 21
Hordeum vulgare	Chi33	L34211	22
Lycopersicon esculentum	Chi9	Q05538/Z15140/S37344	23
Nicotiana tabacum	CBP20[e]	S72424	24
Nicotiana tabacum	Chi	A21091	25
Nicotiana tabacum (cv. Havana)	Chi	A29074/M15173/S20981/S19855	26
Nicotiana tabacum (FB7-1)	Chi	JQ0993/S0828	27
Nicotiana tabacum (cv. Samsun)	Chi	A16119	28
Nicotiana tabacum (cv. Havana)	Chi	P08252/X16939/S08627	27
Nicotiana tabacum (cv. BY4)	Chi	P24091/X51599/X64519//S13322	26,27,29
Nicotiana tabacum (cv. Havana)	Chi	P29059/X64518/S20982	26
Oryza sativum (IR36)	ChiA	L37289	30
Oryza sativum	ChiB	JC2253/S42829/Z29962	31
Oryza sativum	Chi	S39979/S40414/X56787	32
Oryza sativum (cv. Japonicum)	Chi	X56063	33
Oryza sativum (cv. Japonicum)	Chi1	P24626/X54367/S14948	34
Oryza sativum	Chi2	P25765/S15997	35
Oryza sativum (cv. Japonicum)	Chi3	D16223	
Oryza sativum	ChiA	JC2252/S42828	30
Oryza sativum	Chi1	D16221	32
Oryza sativum (IR58)	Chi	U02286	36
Oryza sativum	Chi	X87109	37
Pisum sativum (cv. Birte)	Chi	P36907/X63899	38
Pisum sativum (cv. Alcan)	Chi2	L37876	39
Populus trichocarpa	Chi	S18750/S18751/X59995/P29032	40
Populus trichocarpa (H11-11)	Chi	U01660	41

Phaseolus vulgaris (cv. Saxa)	Chi	A24215/S43926/Jq0965/P36361	42
Phaseolus vulgaris (cv. Saxa)	Chi	P06215/M13968/M19052/A25898	43,44,45
Sambucus nigra	PR-3[f]	Z46948	46
Secale cereale	Chi	JC2071	47
Solanum tuberosum	ChiB1	U02605	48
Solanum tuberosum	ChiB2	U02606	48
Solanum tuberosum	ChiB3	U02607/S43317	48
Solanum tuberosum	ChiB4	U02608	48
Solanum tuberosum (cv. Maris Piper)	WIN-1[g]	P09761/X13497/S04926	49
Solanum tuberosum (cv. Maris Piper)	WIN-2[g]	P09762/X13497/S04927	49
Triticum aestivum	Chi	S38670/X76041	50
Triticum aestivum	WGA-1[h]	P10968/M25536/S09623/S07289	51,52
Triticum aestivum	WGA-2[h]	P02876/M25537/S09624	51,53
Triticum aestivum	WGA-3	P10969/J02961/S10045/A28401	54
Ulmus americana (NPS3-487)	Chi	L22032	55
Urtica dioica	AGL[i]	M87302	56
Vigna unguiculata (cv. Red caloona)	Chi1	X88800	57

[a]NHP : nuclear polyhedrosis virus endochitinase like sequence; Chi : chitinase, [b]anti-microbial peptide, [c]pre-hevein like protein, [d]hevein, [e]chitin-binding protein, [f]pathogenesis related protein, [g]wound-induced protein, [h]wheat germ agglutinin, [i]agglutinin (lectin).

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Table 4
Sources of polysaccharide binding domains

Binding Domain	Proteins Where Binding Domain is Found
Cellulose Binding Domains[1]	β-glucanases (avicelases, CMCases, cellodextrinases)
	exoglucanses or cellobiohydrolases
	cellulose binding proteins
	xylanases
	mixed xylanases/glucanases
	esterases
	chitinases
	β-1,3-glucanases
	β-1,3-(β-1,4)-glucanases
	(β-)mannanases
	β-glucosidases/galactosidases
	cellulose synthases (unconfirmed)
Starch/Maltodextrin Binding Domains	α-amylases[2,3]
	β-amylases[4,5]
	pullulanases
	glucoamylases[6,7]
	cyclodextrin glucotransferases[8-10] (cyclomaltodextrin glucanotransferases)
	maltodextrin binding proteins[11]

Dextran Binding Domains	*(Streptococcal)* glycosyl transferases[12] dextran sucrases (unconfirmed) *Clostridial* toxins[13,14] glucoamylases[6] dextran binding proteins
β-Glucan Binding Domains	β-1,3-glucanases[15,16] β-1,3-(β-1,4)-glucanases (unconfirmed)[17] β-1,3-glucan binding protein
Chitin Binding Domains	chitinases chitobiases chitin binding proteins (see *also* cellulose binding domains) Heivein

[1]Gilkes *et al., Adv. Microbiol Reviews,* (1991) 303-315.
[2]S?gaard *et al., J. Biol. Chem.* (1993) 268:22480.
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[15]Klebl *et al., J. Bacteriol.* (1989) 171:6259.
[16]Watanabe *et al., J. Bacteriol.* (1992) 174:186.
[17]Duvic *et al., J. Biol. Chem.* (1990) :9327.

Thus, and as already stated, the phrase "polysaccharide binding peptide" includes an amino acid sequence which comprises at least a functional portion of a polysaccharide binding region (domain) of a polysaccharidase or a polysaccharide binding protein. The phrase further relates to a polypeptide screened for its cellulose binding activity out of a library, such as a peptide library or a DNA library (e.g., a cDNA library or a display library). By "functional portion" is intended an amino acid sequence which binds to cellulose.

The techniques used in isolating polysaccharidase genes, such as cellulase genes, and genes for cellulose binding proteins are known in the art, including synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. (See, U.S. Pat. Nos. 5,137,819; 5,202,247; 5,340,731; 5,496,934; and 5,837,814). The sequences for several binding domains, which bind to soluble oligosaccharides are known (See, Figure 1 of PCT/CA97/00033, WO 97/26358). The DNAs coding for a variety of polysaccharidases and polysaccharide binding proteins are also known. Various techniques for manipulation of genes are well known, and

include restriction, digestion, resection, ligation, *in vitro* mutagenesis, primer repair, employing linkers and adapters, and the like (see Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, which is incorporated herein by reference).

The amino acid sequence of a polysaccharidase can be used to design a probe to screen a cDNA or a genomic library prepared from mRNA or DNA from cells of interest as donor cells for a polysaccharidase gene or a polysaccharide binding protein gene. By using the polysaccharidase cDNA or binding protein cDNA or a fragment thereof as a hybridization probe, structurally related genes found in other species can be easily cloned and provide a cellulose binding peptide which is expressible in plants according to the present invention. Particularly contemplated is the isolation of genes from organisms that express polysaccharidase activity using oligonucleotide probes based on the nucleotide sequences of genes obtainable from an organism wherein the catalytic and binding domains of the polysaccharidase are discrete, although other polysaccharide binding proteins also can be used (see, for example, Shoseyov, *et al.*, Proc. Nat'l. Acad. Sci. (USA) (1992) 89:3483-3487).

Probes developed using consensus sequences for the binding domain of a polysaccharidase or polysaccharide-binding protein are of particular interest. The β-1,4-glycanases from *C. fimi* characterized to date are endoglucanases A, B, C and D (CenA, CenB, CenC and CenD, respectively), exocellobiohydrolases A and B (CbhA and CbhB, respectively), and xylanases A and D (Cex and XylD, respectively) (see Wong *et al.* (1986) Gene, 44:315; Meinke *et al.* (1991) J. Bacteriol., 173:308; Coutinho *et al.*, (1991) Mol. Microbiol. 5:1221; Meinke *et al.*, (1993) Bacteriol., 175:1910; Meinke *et al.*, (1994) Mol. Microbiol., 12:413; Shen *et al.*, Biochem. J., in press; O'Neill *et al.*, (1986) Gene, 44:325; and Millward-Sadler *et al.*, (1994) Mol. Microbiol., 11:375). All are modular proteins of varying degrees of complexity, but with two features in common: a catalytic domain (CD) and a cellulose-binding domain (CBD) which can function independently (see Millward-Sadler *et al.*, (1994) Mol. Microbiol., 11:375; Gilkes *et al.*, (1988) J. Biol. Chem., 263:10401; Meinke *et al.*, (1991) J. Bacteriol., 173:7126; and Coutinho *et al.*, (1992) Mol. Microbiol., 6:1242). In four of the enzymes, CenB, CenD, CbhA and CbhB, fibronectin type III (Fn3) repeats separate the N-terminal CD from the C-terminal CBD. The CDs of the enzymes come from six of the

families of glycoside hydrolases (see Henrissat (1991) Biochem. J., 280:309; and Henrissat *et al.*, (1993) Biochem. J., 293:781); all of the enzymes have an N- or C-terminal CBD or CBDs (see Tomme *et al.*, Adv. Microb. Physiol., in press); CenC has tandem CBDs from family IV at its N-terminus; CenB and XylD each have a second, internal CBD from families III and II, respectively. Cex and XylD are clearly xylanases; however, Cex, but not XylD, has low activity on cellulose. Nonetheless, like several other bacterial xylanases (see Gilbert *et al.*, (1993) J. Gen. Microbiol., 139:187), they have CBDs. *C. fimi* probably produces other β-1,4-glycanases. Similar systems are produced by related bacteria (see Wilson (1992) Crit. Rev. Biotechnol., 12:45; and Hazlewood *et al.*, (1992) J. Appl. Bacteriol., 72:244). Unrelated bacteria also produce glycanases; *Clostridium thermocellum,* for example, produces twenty or more β-1,4-glycanases (see Beguin *et al.*, (1992) FEMS Microbiol. Lett., 100:523). The CBD derived from *C. fimi* endoglucanase C N1, is the only protein known to bind soluble cellosaccharides and one of a small set of proteins that are known to bind any soluble polysaccharides.

Examples of suitable binding domains are shown in Figure 1 of PCT/CA97/00033 (WO 97/26358), which presents an alignment of binding domains from various enzymes that bind to polysaccharides and identifies amino acid residues that are conserved among most or all of the enzymes. This information can be used to derive a suitable oligonucleotide probe using methods known to those of skill in the art. The probes can be considerably shorter than the entire sequence but should at least be 10, preferably at least 14, nucleotides in length. Longer oligonucleotides are useful, up to the full legnth of the gene, preferably no more than 500, more preferably no more than 250, nucleotides in length. RNA or DNA probes can be used. In use, the probes are typically labeled in a detectable manner, for example, with ^{32}P, ^{3}H, biotin, avidin or other detectable reagents, and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after the unhybridized probe has been separated from the hybridized probe. The hybridized probe is typically immobilized on a solid matrix such as nitrocellulose paper. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Although probes are normally used with a detectable label that allows easy identification, unlabeled oligonucleotides are also useful, both as precursors of labeled probes and for use in methods that provide for direct detection of double-

stranded DNA (or DNA/RNA). Accordingly, the term "oligonucleotide probe" refers to both labeled and unlabeled forms.

Generally, the binding domains identified by probing nucleic acids from an organism of interest will show at least about 40 % identity (including as appropriate allowances for conservative substitutions, gaps for better alignment and the like) to the binding region or regions from which the probe was derived and will bind to a soluble β-1,4 glucan with a K_a of $\geq 10^3\ M^{-1}$. More preferably, the binding domains will be at least about 60 % identical, and most preferably at least about 70 % identical to the binding region used to derive the probe. The percentage of identity will be greater among those amino acids that are conserved among polysaccharidase binding domains. Analyses of amino acid sequence comparisons can be performed using programs in PC/Gene (IntelliGenetics, Inc.). PCLUSTAL can be used for multiple sequence alignment and generation of phylogenetic trees.

In order to isolate the polysaccharide binding protein or a polysaccharide binding domain from an enzyme or a cluster of enzymes that binds to a polysaccharide, several genetic approaches can be used. One method uses restriction enzymes to remove a portion of the gene that codes for portions of the protein other than the binding portion thereof. The remaining gene fragments are fused with expression control sequences to obtain a mutated gene that encodes a truncated protein. Another method involves the use of exonucleases such as *Bal*31 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene deletion methods result in a mutated gene encoding a shortened protein molecule which can then be evaluated for substrate or polysaccharide binding ability.

Any cellulose binding protein or cellulose binding domain may be used in the present invention. The term "cellulose binding protein" ("CBP") refers to any protein or polypeptide which specifically binds to cellulose. The cellulose binding protein may or may not have cellulose or cellulolytic activity. The term "cellulose binding domain" ("CBD") refers to any protein or polypeptide which is a region or portion of a larger protein, said region or portion binds specifically to cellulose. The cellulose binding domain (CBD) may be a part or portion of a cellulase, xylanase or other polysaccharidase, e.g., a chitinase, etc., a sugar binding protein such as maltose binding protein, or scaffoldin such as CbpA of *Clostridium celluvorans, etc.* Many cellulases and hemicellulases (e.g. xylanases and mannases) have the

ability to associate with cellulose. These enzymes typically have a catalytic domain containing the active site for substrate hydrolysis and a carbohydrate-binding domain or cellulose-binding domain for binding cellulose. The CBD may also be from a non-catalytic polysaccharide binding protein. To date, more than one hundred cellulose-binding domains (CBDs) have been classified into at least thirteen families designated I-XIII (Tomme *et al.* (1995) "CelluloseBinding Domains: Classification and Properties", in ACS Symposium Series 618 Enzymatic Degradation and Insoluble Carbohydrates, pp. 142-161, Saddler and Penner eds., American Chemical Society, Washington, D.C. (Tomme I) ; Tomme *et al.* Adv. Microb. Physiol. (1995) 37:1 (Tomme II); and Smant *et al.*, Proc. Natl. Acad. Sci U.S.A. (1998) 95:4906,-4911, all of which are incorporated herein by reference). Any of the CBDs described in Tomme I or II or any variants thereof, any other presently known CBDs or any new CBDs which may be identified can be used in the present invention. As an illustrative, but in no way limiting example, the CBP or CBD can be from a bacterial, fungal, slime mold, or nematode protein or polypeptide. For a more particular illustrative example, the CBD is obtainable from *Clostridium cellulovorans, Clostridium cellulovorans, or Cellulomonas fimi* (e.g., CenA, CenB, CenD, Cex). In addition, the CBD may be selected from a phage display peptide or peptidomimetic library, random or otherwise, using e.g., cellulose as a screening agent. (See Smith Science (1985) 228:1315-1317 and Lam, Nature (1991) 354:82-84). Furthermore, the CBD may be derived by mutation of a portion of a protein or polypeptide which binds to a polysaccharide other than cellulose (or hemicellulose) but also binds cellulose, such as a chitinase, which specifically binds chitin, or a sugar binding protein such as maltose binding protein, rendering said portion capable of binding to cellulose. In any event, the CBD binds cellulose or hemicellulose. Shoseyov and Doi (Proc. Natl. Acad. Sci. USA (1990) 87:2192-2195) isolated a unique cellulose-binding protein (CbpA) from the cellulose "complex" of the cellulolytic bacterium *Clostridium cellulovorans*. This major subunit of the cellulose complex was found to bind to cellulose, but had no hydrolytic activity, and was essential for the degradation of crystalline cellulose. The CbpA gene has been cloned and sequenced (Shoseyov *et al.* Proc. Natl. Acad. Sci. USA (1992) 89:3483-3487). Using PCR primers flanking the cellulose-binding domain of CbpA, the latter was successfully cloned into an overexpression vector that enabled overproduction of the approximately 17 kDa CBD in *Escherichia coli*. The

recombinant CBD exhibits very strong affinity to cellulose and chitin (U.S. Pat. No. 5,496,934; Goldstein *et al.*, J. Bacteriol. (1993) 175:5762; PCT International Publication WO 94/24158, all are incorporated by reference as if fully set forth herein).

In recent years, several CBDs have been isolated from different sources. Most of these have been isolated from proteins that have separate catalytic, i.e., cellulose and cellulose binding domains, and only two have been isolated from proteins that have no apparent hydrolytic activity but possess cellulose-binding activity (Goldstein *et al.* J. Bacteriol. (1993) 175:5762-5768; Morag *et al.* Appl. (1995) Environ. Microbiol. 61:1980-1986).

Recombinant proteins:

Any protein for which a gene is known or can be isolated can be used as the recombinant protein and be fused to the cellulose binding peptide according to the present invention. Advantageously, the recombinant protein is of a commercial value. A non-exhaustive list of recombinant proteins which can be manufactured utilizing the process of the present invention and their uses follows.

Thus, for example, glucoamylases and glucose isomerases are used in the food processing industry to convert starch to high fructose corn syrup.

Another useful class of enzymes are proteinases, which are used for the hydrolysis of high molecular weight proteins and which are further used in combination with detergents in cleaning applications, in leather manufacturing processes, in the food industry, and in the manufacture of alcoholic beverages.

Enzymes known as pectinesterases, and several related enzymes, are used for pectin hydrolysis in the food industry.

A class of enzymes known as lipases are used for the cleavage of ester linkages in triglycerides, and are used both in the food industry and for effluent treatment.

The enzyme beta-galactosidase is used industrially for the hydrolysis of whey lactose.

An enzyme known as thermolysin is used in the production of the artificial sweetener aspartame.

An enzyme known as sulphydryl oxidase is used in the reduction of the cooked flavor of milk.

Enzymes known as catalases are used to remove hydrogen peroxides from milk, cheese, and egg processing, and are further used in the sterilization and oxidation of plastics and rubbers.

Heparinases are useful for the production of heparin and heparan sulfate oligosaccharides.

Other proteins, in addition to enzymes, are those which have affinities to other compounds. For example, bacteria, fungi, plants and animals all contain a large number of proteins that exhibit specific interactions with agents such as metal ions and toxic compounds, and have high affinities for such agents.

A class of proteins known as metalloproteins contain prosthetic groups that bind specifically to metal ions. An example of such a prosthetic group is the porphyrin group in hemoglobin. Some other examples of metal ion binding proteins include parvalbumin, which binds to calcium, and metallothionin, an animal protein that binds large amounts of metal ions, especially zinc. Such metal absorptive proteins could also be used for purification in industrial processes.

It is also envisioned that streams of flowing material could be degraded by microbial enzymes. It is known that certain pollutants, whether natural or synthetic, and certain pesticides and other durable organic compounds in the environment can be degraded (inactivated) or converted into useful compounds by microbial enzymes.

It is known, for example, that some microorganisms, for example *Pseudomonas putida*, possessed dehalogenases that are capable of degrading certain pesticides and herbicides, and rendering them less toxic. Similarly, hydrolysis of organophosphate insecticides have been observed by microbial enzymes.

It is also possible to produce antibodies within plant cells. The antibodies can include monoclonal antibodies or fragments thereof having at least a portion of an antigen binding region, including immunoactive entities such as Fv, F(abl)2, Fab fragments (Harlow and Lane, 1988 Antibody, Cold Spring Harbor), single chain antibodies (U.S. Pat. No. 4,946,778), chimeric or humanized antibodies (Morrison *et al.* Proc. Natl. Acad. Sci. USA (1984) 81:6851; Neuberger *et al.* Nature (1984) 312:604-608) and complementarily determining regions (CDR).

Another class of proteins are those that bind to antibodies, such as protein-A, protein-G, protein-L and their mutants.

It is also possible to produce protein antibiotics or peptides such as lysozyme or therapeutic proteins which might assist in healing processes, for example, certain wound healing peptides, growth factors and hormones. Proteins such as HSA can also be produced.

Another class of proteins include proteins such as agglutinin, zein, silk, elastine proteins as well as COMP, JUN, FOS and other proteins that may form stable protein-protein interactions such as coiled-coil interactions that may be useful for production of protein fibers.

Another example is the production of animal feed enzymes. Phytase from *Aspergillus niger*, for example, increases the availability of phosphorus from feed for monogastric animals by releasing phosphate from the substrate phytic acid, therefore reducing the need for costly phosphorus supplements. A phytase cDNA was constitutively expressed in transgenic tobacco (*Nicotiana tabacum*) plants (Verwoerd *et al.*, Plant. Physiol. (1995) 109:1199-205). Soybean plants transformed with a fungal phytase gene improve phosphorus availability whereas excretion was decreased for broilers. It appears that phytase can improve growth performance of broilers fed low phosphorous diets when provided either as a commercial supplement or in the form of transformed seeds (Denbow *et al.*, Poult. Sci. (1989) 77:878-881).

Other recombinant proteins of interest, will for the most part be mammalian proteins, and will include blood proteins, such as serum albumin, Factor VII, Factor VIIIc, Factor VIIIvW, Factor IX, Factor X, tissue plasminogen factor, Protein C, von Willebrand factor, antithrombin III, erythropoietin, colony stimulating factors, such as G-, M-, GM-, cytokines, such as interleukins 1-11, integrins, addressins, selectins, homing receptors, surface membrane proteins, such as surface membrane protein receptors, T cell receptor units, immunoglobulins (as further detailed above with respect to antibodies), soluble major histocompatibility complex antigens, structural proteins, such as collagen, fibrin, elastin, tubulin, actin, and myosin, growth factor receptors, growth factors, growth hormone, cell cycle proteins, vaccines, fibrinogen, thrombin, cytokines and hyaluronidase. Additional examples include chymosin, polymerases, saccharidases, dehydrogenases, nucleases, oxido reductases such as fungal peroxidases and lactases, xylanases, rennin, horse radish peroxidase, amylases and soil remediation enzymes.

The genes encoding all of the above listed proteins have been isolated and as such these proteins are readily available for recombinant

expression and production according to the teachings of the present invention. It will be appreciated that new genes encoding an ever growing spectrum of proteins are continuously discovered and isolated, rendering such genes available for molecular manipulation and recombinant expression. There is thus no intention to limit the recombinant protein produced utilizing the method of the present invention to any specific protein or list of proteins.

Cellulose binding peptide-recombinant protein fusions:

The fusion of two proteins for which genes has been isolated is well known and practiced in the art. Such fusion involves the joining together of heterologous nucleic acid sequences, in frame, such that translation thereof results in the generation of a fused protein product or a fusion proteins. Methods, such as the polymerase chain reaction (PCR), restriction, nuclease digestion, ligation, synthetic oligonucleotides synthesis and the like are typically employed in various combinations in the process of generating fusion gene constructs. One ordinarily skilled in the art can readily form such constructs for any pair or more of individual proteins. Interestingly, in most cases where such fusion or chimera proteins are produced, and in all cases where one of the proteins was a cellulose binding peptide, both the former and the latter retained their catalytic activity or function.

For example, Greenwood *et al.* (1989, FEBS Lett. 224:127-131) fused the cellulose binding region of *Cellulomonas fimi* endoglucanase to the enzyme alkaline phosphatase. The recombinant fusion protein retained both its phosphatase activity and the ability to bind to cellulose. For more descriptions of cellulose binding fusion proteins, see U.S. Patent No. 5,137,819 issued to Kilburn *et al.*, and U.S. Patent No. 5,719,044 issued to Shoseyov *et al.* both incorporated by reference herein. See also U.S. Pat. No. 5,474,925. All of which are incorporated herein by reference.

The recombinant protein immobilized via its fused counterpart to the cellulosic matter can be released from the plant derived cellulosic matter by cleavage thereof, e.g., by proteolysis, using either a nonspecific general protease such as proteinase K or trypsin, or a specific protease as further detailed hereinunder. For example, release can be effected by treatment with proteinase K at a concentration of about 50 μg/ml for about 20 minutes at about 37 °C (Din *et al.* Bio/Technology (1991) 9:1096-1099).

Inclusion of a dedicated cleavage site:

According to a preferred embodiment of the present invention the fusion protein includes the recombinant protein and the cellulose binding

peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

As used herein in the specification and in the claims section that follows, the phrase "unique amino acid sequence recognizable and digestible by a protease" includes a protease recognition sequence which is both recognizable and readily accessible to a protease. Thus, the unique sequence can be a solitary sequence (i.e., which does not appear in the recombinant protein and optionally also not in the cellulose binding peptide) or alternatively, the sole sequence of several similar sequences which is not sequestered from the protease due to the tertiary structure of the recombinant protein and optionally the cellulose binding peptide. In both these cases proteolysis will release the recombinant protein from the fusion protein cellulosic matter complex.

As used herein in the specification and in the claims section that follows, the phrase "controllable intervening protein sequence" includes unique amino acid sequences capable of excision from or cleavage of a peptide under predetermined conditions in cis or in trans, e.g., increase in temperature, exposure to light, unblocking of amino acid residues by dephosphorylation and treatment with chemical reagents or deglycosylation, examples include proteolysis effected under acidic conditions (HCl, e.g., to cleave between Asp and Pro) and proteolysis effected by a proteolysing reagent, such as CNBr to cleave downstream of Met, all as known in the art and/or as further described in U.S. Pat. No. 5,834,247, which is incorporated herein by reference.

Thus, according to an aspect of the present invention there is provided a composition of matter comprising (a) a plant derived cellulosic matter; and (b) a fusion protein including a recombinant protein and a cellulose binding peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, wherein the fusion protein is complexed to the plant derived cellulosic matter by affinity binding via the cellulose binding peptide.

Nucleic acid molecules which can be used according to preferred embodiments of the present invention to express the fusion protein in plant cells would therefore include a heterologous nucleic acid sequence including (i) a first sequence encoding a cellulose binding peptide; (ii) a second sequence encoding a recombinant protein, wherein the first and

second sequences are joined together in frame in either orientation; and (iii) a third sequence encoding a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, the third sequence is between and in frame with the first and second sequences.

Thus, specific cleavage can be used to release the recombinant protein from the fusion protein cellulosic matter complex. For example, one can include a protease recognition site or a chemical cleavage site between the recombinant protein and the cellulose binding peptide. Examples of recognition sites include those for collagenase, thrombin, enterokinase, and Factor X_a which are cleaved specifically by the respective enzymes. Chemical cleavage sites sensitive, for example, to low pH or cyanogen bromide, can also be used.

Where cleavage is used, the recombinant protein can be cleaved readily from the cellulosic matter by the use of a protease specific for a sequence present therebetween and the cellulose binding peptide.

It will be appreciated in this respect that four main classes of specific proteases are known, including (i) cysteine proteases, including cathepsin B and L; (ii) aspartyl protease cathepsin D; (iii) serine proteases including plasmin, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), and (iv) matrix metalloproteinases (MMPs), including collagenases, gelatinases A and B (MMP2 and MMP9) and stromelysin (MMP3). Members of these protease families are commercially available and their recognition sequences known. As such, these proteases can be used to implement the step of releasing the recombinant protein from the plant derived cellulosic matter while implementing the process according to the present invention.

Genetically modified plant material:

According to an aspect of the present invention there is provided a nucleic acid molecule comprising (a) a promoter sequence for directing protein expression in plant cells; and (b) a heterologous nucleic acid sequence as further detailed herein, wherein, the heterologous nucleic acid sequence is down stream the promoter sequence, such that expression of the heterologous nucleic acid sequence is effectable by the promoter sequence. Such a nucleic acid molecule needs to be effectively introduced into plant cells, so as to genetically modify the plant.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledenous plants (Potrykus, I., Annu. Rev.

Plant. Physiol., Plant. Mol. Biol. (1991) 42:205-225; Shimamoto *et al.*, Nature (1989) 338:274-276). The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:

(i) *Agrobacterium*-mediated gene transfer: Klee *et al.* (1987) Annu. Rev. Plant Physiol. 38:467-486; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) direct DNA uptake: Paszkowski *et al.*, in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. *et al.* (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang *et al.* Plant Cell Rep. (1988) 7:379-384. Fromm *et al.* Nature (1986) 319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein *et al.* Bio/Technology (1988) 6:559-563; McCabe *et al.* Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus *et al.*, Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; or by the direct incubation of DNA with germinating pollen, DeWet *et al.* in Experimental Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719.

The *Agrobacterium* system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. Horsch *et al.* in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. The *Agrobacterium* system is especially viable in the creation of transgenic dicotyledenous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transgenic plant be produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant, e.g., a reproduction of the fusion protein. Therefore, it is preferred that the transgenic plant be regenerated by micropropagation which provides a rapid, consistent reproduction of the transgenic plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are

produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transgenic plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable marker; and, for *Agrobacterium* transformations, T DNA sequences for *Agrobacterium*-mediated transfer to plant chromosomes. Where the heterologous sequence is not readily amenable to detection, the construct will preferably also have a selectable marker gene suitable for determining if a plant cell has been transformed. A general review of suitable markers for the members of the grass family is found in Wilmink and Dons, Plant Mol. Biol. Reptr. (1993) 11:165-185.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are also recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome.

Suitable prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The constructs of the subject invention will include an expression cassette for expression of the fusion protein of interest. Usually, there will be only one expression cassette, although two or more are feasible. The recombinant expression cassette will contain in addition to the heterologous sequence one or more of the following sequence elements, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

Viral infected plant material:

Viruses are a unique class of infectious agents whose distinctive features are their simple organization and their mechanism of replication. In fact, a complete viral particle, or virion, may be regarded mainly as a block of genetic material (either DNA or RNA) capable of autonomous

replication, surrounded by a protein coat and sometimes by an additional membranous envelope such as in the case of alpha viruses. The coat protects the virus from the environment and serves as a vehicle for transmission from one host cell to another.

Viruses that have been shown to be useful for the transformation of plant hosts include CaV, TMV and BV. Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. *et al.*, Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, is described in WO 87/06261.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as by Dawson, W. O. *et al.*, Virology (1989) 172:285-292; Takamatsu *et al.* EMBO J. (1987) 6:307-311; French *et al.* Science (1986) 231:1294-1297; and Takamatsu *et al.* FEBS Letters (1990) 269:73-76.

When the virus is a DNA virus, the constructions can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as in U.S. Pat. No. 5,316,931

In one embodiment, a plant viral nucleic acid is provided in which the native coat protein coding sequence has been deleted from a viral nucleic acid, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native

coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral nucleic acid, and ensuring a systemic infection of the host by the recombinant plant viral nucleic acid, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native nucleic acid sequence within it, such that a fusion protein is produced. The recombinant plant viral nucleic acid may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or nucleic acid sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) nucleic acid sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one nucleic acid sequence is included. The non-native nucleic acid sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral nucleic acid is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral nucleic acid is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral nucleic acid. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native nucleic acid sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that said sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral nucleic acid is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral nucleic acid to produce a recombinant plant virus. The recombinant plant viral nucleic acid or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral

nucleic acid is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) in the host to produce the desired fusion protein.

Fusion protein compartmentalization - signal peptides:

As already mentioned hereinabove, compartmentalization of the fusion protein is an important feature of the present invention because it allows undisturbed plant growth. Thus, according to one aspect of the present invention, the fusion protein is compartmentalized within cells of the plant or cultured plant cells, so as to be sequestered from cell walls of the cells of the plant or cultured plant cells.

The fusion protein can be compartmentalized within a cellular compartment, such as, for example, the cytoplasm, endoplasmic reticulum, golgi apparatus, oil bodies, starch bodies, chloroplastids, chloroplasts, chromoplastids, chromoplasts, vacuole, lysosomes, mitochondria or the nucleus.

Accordingly, the heterologous sequence used while implementing the process according to this aspect of the present invention includes (i) a first sequence encoding a cellulose binding peptide; (ii) a second sequence encoding a recombinant protein, wherein the first and second sequences are joined together in frame; and (iii) a third sequence encoding a signal peptide for directing a protein to a cellular compartment, the third sequence being upstream and in frame with the first and second sequences.

The following provides description of signal peptides which can be used to direct the fusion protein according to the present invention to specific cell compartments.

It is well-known that signal peptides serve the function of translocation of produced protein across the endoplasmic reticulum membrane. Similarly, transmembrane segments halt translocation and provide anchoring of the protein to the plasma membrane, see, Johnson *et al.* The Plant Cell (1990) 2:525-532; Sauer *et al.* EMBO J. (1990) 9:3045-3050; Mueckler *et al.* Science (1985) 229:941-945. Mitochondrial, nuclear, chloroplast, or vacuolar signals target expressed protein correctly into the corresponding organelle through the secretory pathway, see, Von Heijne, Eur. J. Biochem. (1983) 133:17-21; Yon Heijne, J. Mol. Biol. (1986) 189:239-242; Iturriaga *et al.* The Plant Cell (1989) 1:381-390; McKnight *et al.*, Nucl. Acid Res. (1990) 18:4939-4943; Matsuoka and Nakamura, Proc. Natl. Acad. Sci. USA (1991) 88:834-838. A recent book by Cunningham and Porter (Recombinant proteins from plants, Eds. C. Cunningham and

A.J.R. Porter, 1998 Humana Press Totowa, N.J.) describe methods for the production of recombinant proteins in plants and methods for targeting the proteins to different compartments in the plant cell. In particular, two chapters therein (14 and 15) describe different methods to introduce targeting sequences that results in accumulation of recombinant proteins in compartments such as ER, vacuole, plastid, nucleus and cytoplasm. The book by Cunningham and Porter is incorporated herein by reference. Presently, the preferred site of accumulation of the fusion protein according to the present invention is the ER using signal peptide such as Cel 1 or the rice amylase signal peptide at the N-terminus and an ER retaining peptide (HDEL, SEQ ID NO:1; or KDEL, SEQ ID NO:2) at the C-terminus.

Promoters and control of expression:

Any promoter which can direct the expression of the fusion protein according to the present invention can be utilized to implement the process of the instant invention, both constitutive and tissue specific promoters. According to presently preferred embodiment the promoter selected is constitutive, because such a promoter can direct the expression of higher levels of the fusion protein. In this respect the present invention offers a major advantage over the teachings of U.S. Pat. No. 5,474,925 in which only tissue specific and weak promoters can be employed because of the detelerious effect of the fusion protein described therein on cell wall development. The reason for which the present invention can utilize strong and constitutive promoters relies in the compartmentalization and sequestering approach which prohibits contact between the expressed fusion protein and the plant cell walls which such walls are developing.

Constitutive and tissue specific promoters, CaMV35S promoter (Odell *et al.* Nature (1985) 313:810-812) and ubiquitin promoter (Christensen and Quail, Transgenic research (1996) 5:213-218) are the most commonly used constitutive promoters in plant transformations and are the preferred promoters of choice while implementing the present invention.

In corn, within the kernel, proteins under the ubiquitin promoters, are preferentially accumulated in the germ (Kusnadi *et al.*, Biotechnol. Bioeng. (1998) 60:44-52). The amylose-extender (Ae) gene encoding starch-branching enzyme IIb (SBEIIb) in maize is predominantly expressed in endosperm and embryos during kernel development (Kim *et al.* Plant. Mol. Biol. (1998) 38:945-956). A starch branching enzyme (SBE) showed promoter activity after it was introduced into maize endosperm suspension cells by particle bombardment (Kim *et al.* Gene (1998) 216:233-243). In

transgenic wheat it has been shown that a native HMW-GS gene promoter can be used to obtain high levels of expression of seed storage and, potentially, other proteins in the endosperm (Blechl and Anderson, Nat. Biotechnol. (1996) 14:875-9). Polygalacturonase (PG) promoter was shown to confer high levels of ripening-specific gene expression in tomato (Nicholass *et al.* Plant. Mol. Biol. (1995) 28:423-435). The ACC oxidase promoter (Blume and Grierson, Plant. J. (1997) 12:731-746) represents a promoter from the ethylene pathway and shows increased expression during fruit ripening and senescence in tomato. The promoter for tomato 3-hydroxy-3-methylglutaryl coenzyme A reductase gene accumulates to high level during fruit ripening (Daraselia *et al.* Plant. Physiol. (1996) 112:727-733). Specific protein expression in potato tubers can be mediated by the patatin promoter (Sweetlove *et al.* Biochem. J. (1996) 320:487-492). Protein linked to a chloroplast transit peptide changed the protein content in transgenic soybean and canola seeds when expressed from a seed-specific promoter (Falco *et al.* Biotechnology (NY) (1995) 13:577-82). The seed specific bean phaseolin and soybean beta-conglycinin promoters are also suitable for the latter example (Keeler *et al.* Plant. Mol. Biol. (1997) 34:15-29). Promoters that are expressed in plastids are also suitable in conjunction with plastid transformation.

Each of these promoters can be used to implement the process according to the present invention.

Thus, the plant promoter employed can a constitutive promoter, a tissue specific promoter, an inducible promoter or a chimeric promoter.

Examples of constitutive plant promoters include, without being limited to, CaMV35S and CaMV19S promoters, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

Examples of tissue specific promoters include, without being limited to, bean phaseolin storage protein promoter, DLEC promoter, PHSβ promoter, zein storage protein promoter, conglutin gamma promoter from soybean, AT2S1 gene promoter, ACT11 actin promoter from *Arabidopsis*, napA promoter from *Brassica napus* and potato patatin gene promoter.

The inducible promoter is a promoter induced by a specific stimuli such as stress conditions comprising, for example, light, temperature, chemicals, drought, high salinity, osmotic shock, oxidant conditions or in case of pathogenicity and include, without being limited to, the light-

inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21 active in high salinity and osmotic stress, and the promoters hsr303J and str246C active in pathogenic stress.

Expression follow up:

Expression of the fusion protein can be monitored by a variety of methods. For example, ELISA or western blot analysis using antibodies specifically recognizing the recombinant protein or its cellulose binding peptide counterpart can be employed to qualitatively and/or quantitatively monitor the expression of the fusion protein in the plant. Alternatively, the fusion protein can be monitored by SDS-PAGE analysis using different staining techniques, such as, but not limited to, coomasie blue or silver staining. Other methods can be used to monitor the expression level of the RNA encoding for the fusion protein. Such methods include RNA hybridization methods, e.g., Northern blots and RNA dot blots.

Binding of the fusion protein to the plant derived cellulosic matter:

When sufficient expression has been detected, binding of the fusion protein to the plant derived cellulosic matter is effected. Such binding can be achieved, for example, as follows. Whole plants, plant derived tissue or cultured plant cells are homogenized by mechanical method in the presence or absence of a buffer, such as, but not limited to, PBS. The fusion protein is therefore given the opportunity to bind to the plant derived cellulosic matter. Buffers that may include salts and/or detergents at optimal concentrations may be used to wash non specific proteins from the cellulosic matter.

Extraction and purification:

In general, a recent book by Cunningham and Porter (Recombinant proteins from plants, Eds. C. Cunningham and A.J.R. Porter, 1998 Humana Press Totowa, N.J.) describes methods for the production of recombinant proteins in plants including methods for extraction of the proteins from the plants. The methods used herein for extraction of proteins from plants are similar, however the ability of the fusion protein to bind to cellulose dictates its fate, unless extraction is done under condition in which the cellulose binding peptide do not bind to cellulose, for example, pH higher than 10 (for most CBDs) or high concentration of glucose or cellobiose (200 mM or higher) for family IX CBDs. If the initial extraction is conducted under conditions that prevent binding, the supernatant is

cleared from the cellulosic matter and then the solution is brought by either dilution, dialysis or pH correction, if necessary, to a condition that enables binding, after which cellulose is added in a batch or the solution is loaded on a cellulose column. Cellulose affinity purification is conducted as described, for example, in U.S. Pat. Nos. 5,719,044; 5,670,623; 5,856,021; 5,137,819; 5,202,247; 5,340,731; and 5,474,925; and U.S. Pat. applications Nos. 08/788,621; and 08/788,622; EP 0 381 719 B1, and EP application No. 93907724.4. Alternatively, the extraction solution provides conditions that favor binding to the plant derived cellulosic matter.

In any case, while the fusion protein is bound to cellulose, further whases can be employed for further removal of unbound proteins, conditions which dissociate such binding or proteolytic cleavage can be used to isolate the fusion protein itself, or proteolytic cleavage can be used to isolate the recombinant protein, all as further detailed hereinabove.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which is not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion. Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the

information contained therein, as well as that contained in the Manual by Sambrok *et al.*, is incorporated herein by reference.

MATERIALS, CONSTRUCTS AND EXPERIMENTAL METHODS

Enzymes and Chemicals:

Chemicals were purchased from Sigma Israel Chemicals Ltd. (Rehovot, Israel) unless otherwise stated. Restriction enzymes were purchased from MBI Fermentas, Inc. (Amherst NY, USA) and Taq DNA polymerase was purchased from Promega Corp. (Madison, WI, USA).

Plasmids and Bacteria:

The ligation mixture of each cloning procedure was transformed into *E. coli* strain XL1-blue (Stratagene) competent cells. The bacteria were plated on LB-agar plates including 100 μg/ml ampicillin in the case of the pBlueScript and pUC plasmids, or 50 μg/ml kanamycin in the case of the shuttle vectors. Positive clones were verified by restriction analysis and sequencing.

Buffers and Media:

LB (Luria-Broth) - 1% bacto-tryptone, 0.5% yeast extract and 1% NaCl; PBS (Phosphate Buffered Saline) - 20 mM KH_2PO_4, 150 mM NaCl pH 7.2; PBS-T – PBS with 0.1% Tween 20.

Plant Materials and Growth Conditions:

Nicotiana tabaccum-SR1 (tobacco) plants were grown at 24-25 °C under a 16 h photoperiod, using cool-white fluorescent light (50-60 μE m^{-2} S^{-1}).

Construction of ProtL-cex:

A vector containing the class-I patatin gene B33 promoter (Olesinski *et al.*, 1996, Plant Phisiol. 111:541-550), fused to the cell signal sequence (Shani *et al.*, 1997, Plant Mol. Biol. 34:837-842), protein-L (hereinafter ProtL, Nilson *et al.*, 1993, J. Immunol. Meth. 164:33-40), CBD cex sequence (Gilkes *et al.*, 1991, Microbiol. Rev. 55:303-315) and HDEL (SEQ ID NO:1) retaining peptide sequence was constructed as follows.

A DNA fragment encoding the cell signal peptide (nucleotide 1-105 of SEQ ID NO:3) was generated by PCR using the following primers: No. 1 (BglII) 5'-AAAAAGATCTATGGCGCGAAAATCACTAA-3' (SEQ ID NO:4) and No. 2 (XbaI) 5'-AAAATCTAGATTTACGGAGAGCGTCGCG-3' (SEQ ID NO:5). A DNA fragment encoding the ProtL-CBDcex (nucleotides 3-1280 of SEQ ID NO:6) was generated by PCR using the

following primers: No. 3 (XbaI) 5'-AAAATCTAGAATGGCG GCGGTAGAAAATAAAG-3' (SEQ ID NO:7); and No. 4 (HDEL, Stop and SalI) 5'-AAAAGTCGACTTAAAGTTCATCATGCTCGACGCC GACCGTGCA-3' (SEQ ID NO:8). The two fragment were digested with BglII, XbaI and SalI and ligated in one step into pUC19 (New England Biolabs, Beverly, Massachusetts) that was pre digested with BamHI and SalI. The primer for the c-terminal end of the ProtL-CBD contained the retaining peptide sequence HDEL (SEQ ID NO:1) and a stop codon in frame. The DNA containing the Cell Signal-ProtL-CBD-HDEL (hereinafter, ProtL-CBD) fusion was excised using SmaI and SalI and was subcloned into the SmaI and SalI sites of the binary vector Bin19 (Bevan, 1984, Nuc. Acid Res. 12:8711-8721) under the class-I patatin gene B33 promoter (Olesinski *et al.*, 1996, Plant Phisiol. 111:541-550).

Construction of ProtL-cexNG (Non-Glycosylated):

The non-glycosilated form of CBDcex (CBD Technologies Ltd.) was cloned into the Cell-ProtL-cex vector constructed as described above, thus replacing the CBDcex with CBDcexNG. Cloning was performed using the following PCR primers: Primer E, 5'-AAAAACTAGTGCTAGCGG TCCAGCCGGC-3' (SEQ ID NO:9) which is a forward primer containing an *Spe*I restriction site and primer F, 5'-AAAAGTCGACTTA AAGTTCATCATGTCCAACGGTGCAAGGGGC-3' (SEQ ID NO:10) which is a reverse primer containing the ER retaining peptide sequence (HDEL), a stop codon and a *Sal*1 restriction site. The resultant 360 bp PCR product was digested with *Spe*I and *Sal*I and ligated into Cell-ProtL-cex predigested with the same enzymes. Positive clones were sequenced for verification and designated pUC19-cell-ProtL-cexNG-HDEL. Figure 2a shows the coding sequence (SEQ ID NO:11) and the encoded protein (SEQ ID NO:12) of construct pUC19-cell-ProtL-cexNG-HDEL, which is schematically presented in Figure 2b.

Construction of the Shuttle Vector Containing a 35S-Ω Promoter:

The Cell-Protl-cexNG insert which was obtained by generated by digesting the pUC19-cell-ProtL-cexNG-HDEL vector with *Sma*I and *Sph*I (*Pae*I) was sub cloned into a Cd vector containing the CaMV 35S-Ω promoter. To ligate the Cell-Protl-cexNG-HDEL insert, the Cd vector was digested with *Sal*I and the overhang tail was blunted by a fill-in reaction using the Klenow fragment. The vector was then digested with *Sph*I.

The resultant 35SΩ-cell-ProtL-cexNG-HDEL vector and the pBI101 shuttle vector were digested via *Sma*I and *Sac*I and co-ligated to generate pBI-35SΩ-cell-ProtL-cexNG-HDEL.

Construction of cex-Fx and Tma-Fx:

A CBDcex-Fx insert provided in a pBluescript II KS plasmid (pBS-cex-Fx) was obtained from Prof. Douglas Kilburn, Department of Microbiology and Immunology, Biotechnology Laboratory, The University of British Columbia, Vancouver. An ER retention peptide HDEL encoding sequence was ligated at the C-terminus of CBDcex-Fx as followed: Forward primer 49, 5'-CTAGTCATGATGAACTTTAAGAGCT-3' (SEQ ID NO:13) and reverse primer 50, 5'-CTTAAAGTTCATCATGA-3' (SEQ ID NO:14) were mixed together at equi-molar ratios under denaturing conditions (94 ° C). The mixture was then allowed to cool to RT in order to allow annealing. The annealed primers were ligated into pBS-cex-Fx which was predigested with *Spe*I and *Sac*I and the ligation mixture was used to transform XL1 blue competent cells. Positive clones were sequenced for verification and designated as pBS-cex-Fx-HDEL.

A Cell signal peptide encoding sequence was cloned into the N-terminus of pBS-cex-Fx-HDEL. The Cell signal peptide encoding sequence was PCR amplified from pMH04 (Shani, Z., Dekel, M., Tsabary, G. and Shoseyov, O. (1997) Cloning and characterization of elongation specific endo-1,4-β-glucanase (cell) from Arabidopsis thaliana. Plant Molec. Biol. 34: 837-842.) using the following primers: Forward primer 51, 5'-AAAACCCGGGATGGCGCGAAAATC-3' (SEQ ID NO:15), containing a *Sma*I restriction site, and reverse primer 52, 5'-AAAAGACGTCTTAC GGAGAGCGTCGCGGTAATC-3' (SEQ ID NO:16) containing an *Aat*II restriction site. The resulting 115 bp PCR product was digested with *Sma*I and *Aat*II and ligated into pBS-cex-Fx-HDEL. The ligation mixture was used to transform *E. coli* XL1 Blue competent cells. Positive clones were verified via sequencing and designated as pBS-Sig-cex-Fx-HDEL. Figure 3a shows the coding sequence (SEQ ID NO:17) and the encoded protein (SEQ ID NO:18) of construct pBS-Sig-cex-Fx-HDEL, which is schematically presented in Figure 3b.

Replacing CBDcex with CBDTma:

CBDTma was PCR amplified from pET-CBDTma (Alam, M., Boraston, A.B., Kormos, J., Tomme, P. and Kilburn, D.G. Properties of the C-terminal family 9 cellulose-binding module of xylanase A from the hyperthermophilic bacterium Termatoga maritime, Submitted) using

forward primer 53, 5'-AAAAGACGTCGGCTAGCGGAATAATGGTA GCG-3', (SEQ ID NO:19), containing an *Aat*II restriction site, and reverse primer 54, 5'-AAAAACGCGTTGGGGATGGGGTCGGAC-3' (SEQ ID NO:20), containing an *Mlu*I restriction site. The resultant 600 bp PCR product was digested with *Aat*II and *Mlu*I and ligated intpBS-Sig-cex-Fx-HDEL that was predigested with the same enzymes. The ligation mixture was used to transform *E.coli* XL1 Blue competent cells. Positive clones were verify via sequencing and designated as pBS-Sig-Tma-Fx-HDEL. Figure 4a shows the coding sequence (SEQ ID NO:21) and the encoded protein (SEQ ID NO:22) of construct pBS-Sig-Tma-Fx-HDEL, which is schematically presented in Figure 4b.

Shuttle Vector cloning:

A PJD-330 vector which contained the CaMV-35S-Ω promoter (a kind donation from Prof. Gadi Galili, The Weizzmann Institute, Rehovot, Israel,Shaul, O. and Galili, G. (1992) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of Escherichia coli. Plant Phisiol. 100: 1157-1163.) was digested with *Hind*III and *Sal*I. A 500 bp fragment was rescued and ligated into pBI101 (Clontech Laboratories Inc. Palo Alto, California, USA) predigested with the same enzymes. The ligation mixture was used to transform *E.coli* XL1 Blue competent cells and verified positive clones were designated pBI-35S-Ω.

To clone CBDcex-Fx and CBDTma-Fx into the above described shuttle vector, pBS-Sig-cex-Fx-HDEL and pBS-Sig-Tma-Fx-HDEL were each digested with *Sma*I and *Sac*I and respective 1.3 and 1.5 kb fragments were rescued from these vectors were each ligated into pBI-35S-Ω predigested with the same enzymes. The ligation mixture was used to transform *E. coli* XL1 Blue competent cells and positive clones were designated pBI-Sig-cex-Fx-HDEL and pBI-Sig-Tma-Fx-HDEL respectively.

Plant Transformation:

The above described constructs were introduced into disarmed LB 4404 *Agrobacterium tumefaciens* by triparental mating (An, 1987, Meth. Enzymol. 153: 292-305) and leaf-disc transformation was performed with Nicotiana tabaccum-SR1 plants as described previously (DeBlock et al., 1984, EMBO J. 3:1681). Regenerated transgenic plants were selected on kanamycin containing growth media and analyzed via PCR for the presence of exogenic sequences as described below. Positive isolates were grown in

a tissue culture room or in a greenhouse and F_0 plants from independent transformation events were used for the protein purification assays.

Detection of Transgenic Plants by PCR from Chromosomal DNA:

DNA was extracted from leaves of Nicotiana tabaccum-SR1 (tobacco) as described by Doyle and Doyle (1987, Phytochem. Bull. 19:11-15) and the ProtL-cex sequence PCR amplified from the transgenic plant DNA isolated using the following primers: Forward primer 1, 5'-AAAACCATGGCGGCGGTAGAAAATAAAG-3' (SEQ ID NO:23) and reverse primer 2, 5'-AAAAGGATCCCTTCTGGTTTTTCGTCAAC -3' (SEQ ID NO:24).

In a similar manner, cex-Fx and Tma-Fx sequences were also PCR amplified from transgenic plant DNA by using the following primers: Forward primer 3, 5'-AAAACCCGGGATGGCGCGAAAATC-3' (SEQ ID NO:25) and reverse primer 4, 5'- TGCGTTCCAGGGTCTGTTTCC-3' (SEQ ID NO:26). The PCR reaction mixture included 2.5 µl 10X Taq polymerase buffer (Promega, Madison, WI), dNTP mix (0.2 mM each nucleotide), 1.5 mM $MgCl_2$, 10 pmol of each primer, 1 unit Taq DNA polymerase (Promega, Madison, WI) and ddH_2O to a final volume of 25 µl. Mineral oil (25 µl) was added to the mixture to prevent evaporation during cycling. The PCR program included 35 cycles of: denaturation at 95 °C for 60 sec, annealing at 55 °C - 65 °C for 1 min and extension at 72 °C for 1-2 min. The resulting amplified fragment was purified from an agarose-TBE gel.

Purification of ProtL-cexNG:

As outlined in Figure 5, purification of ProtL-cex was conducted as follow: 0.5 gram of tobacco leaf was grounded in liquid nitrogen. The grounded material was resuspended in PBS-T containing 2 mM PMSF, 5 mM EDTA and 2 mM DTT and the mixture was incubated at 4 °C for 1 hour with inversion to allow binding of the ProtL-cex to the cell wall fraction. The mixture was centrifuged, and the supernatant and pellet recovered separately. The pellet was washed 3 times with PBS-T and the washes were combined to the supernatant fraction which was then reacted with 10 mg Avicel 200. The washed cellulose pellet was washed 3 more times with PBS-T. The pellet and supernatant fractions were each separated on SDS-PAGE, and immunobloted using either anti CBDcex Ab or mouse IgG reagent grades primary antibodies and appropriate secondary antibodies conjugated to HRP.

Purification of CBDcex-Fx and CBDTma-Fx:

Purification of CBDcex-Fx and CBDTma-Fx was conducted as described above. Analysis of the resultant pellet and supernatant fractions was conducted using the anti-Fx, anti-CBDcex or anti-CBDTma primary antibodies and appropriate secondary antibodies conjugated to HRP.

EXPERIMENTAL RESULTS

Approximately 30 independent transgenic tobacco plants (F_0, parental generation) were prepared from each of the ProtL-cex and Tma-Fx transformant lines. Conformation of the presence of a transgene was conducted by kanamycin resistance and PCR analysis with specific primers as described above. The primers detected a 0.95 kb fragment in Prot-L transgenic plants (Figure 6), and 0.55 kb and 0.8 kb fragments from cex-Fx (Figures 7a-7b) and Tma-Fx transgenic plants (Figures 8a-b), respectively. In all cases, the binary vector was used as a positive control. Expression of the cellulose binding domain (CBD) in the ProtL-cex and Tma-Fx transgenic plants was confirmed via western blot analysis.

Detection and Purification of ProtL-cexNG:

Of the positive transformants identified via PCR amplification, four plants (1, 2, 5 and 15) expressed ProtL-CBD to a detectable level. The total protein from leaf tissue of transformed plants was extracted and allowed to bind to the cell wall cellulose. The unbound protein in the soluble fraction of the total protein was allowed to bind to exogenous cellulose as described in materials and methods. Western blot analysis of both fractions of the cell wall and the cellulose displayed a difference in the amount of ProtL-CBD present. ProtL-CBD was not detected in the cell wall fraction whereas in the cellulose fraction, a unique band was detected (Figure 9a). The detected ProtL-CBDcexNG was of a higher molecular weight (MW) as compared to the bacterial ProtL-cex which was used as a positive control. This may be due to glycosilation of the plant expressed protein. The ability of the ProtL-cexNG to bind cellulose and the ability of protein L to bind mouse IgG even following gel analysis (Figure 9b), confirmed that the two bi-functional fusion proteins are active. The anti-CBDcex as well as the mouse IgG western blots detected non-specific bands in the cell wall fraction. These non-specific bands appeared in the transgenic and wt plants, and represent a protein with a MW different than that expected for ProtL-cex.

By correlating the brightness of the signal specific band observed on the western blot with bands of ProtL-CBD isolated from *E.coli.*, the amount of ProtL-CBDcexNG accumulated in the transformed plant tissue was approximated to be 1 μg fusion protein per gram of plant tissue.

Detection and Purification of cex-Fx:

Cex-Fx transformants were examined for their ability to express cex-Fx. Two transformant lines (5 and 12) expressed the protein to a detectable level. CBDcex-Fx was detected in the cell wall and cellulose fractions (prepared as described above) in equal amounts (Figures 10a-b). The amount of cex-Fx produced in the plant tissue was approximated at 5 μ g fusion protein per gram of plant tissue. As is evident from Figures 10a-b, the expressed fusion protein is of a higher MW then control CBDcex-Fxa which is expressed in mammalian cells. This shift in MW could be a result of inefficient processing of the protein, at the kex2 and Fx cleavage sites.

Detection and Purification of Tma-Fx:

Tma-Fx transformants were examined for their ability to express Tma-Fx. Four transformants (11, 14, 17 and 19) expressed the protein to a detectable level. The expressed CBDTma-Fx was found only in the exogenous cellulose fraction (prepared as described above) indicating that the CBDTma did not bind cell wall in the transformant plants (Figures 11a-d). This could be due tothe high concentration of endogenous soluble sugars such as glucose, and cellobiose, which prevent the CBDTma from binding to the cellulose matrix. The soluble sugars in the supernatant fraction are diluted by the repeated washes and as such, the CBDTma accumulated in this fraction is able to bind with exogenously added cellulose.

Further support for this theory can be found in the results of transformant 19 (Figures 11c-d). The amount of tissue recovered from this plant for extraction was significantly lower (4-10 times) then that recovered from the other plants. Since the final volume of the samples was equal, the sugar concentration in the sample extracted from transformant 19 was lower, resulting in binding to the cell wall fraction.

The expressed fusion protein appeared to be of a higher MW then CBDcex-Fxa expressed in mammalian cells (positive control). This shift in MW could be a result of inefficient processing of the protein, at the kex2 and Fx cleavage sites. The amount of Tma-fx accumulated in the transformant plant tissue was approximately 5 μg of fusion protein per gram of plant tissue.

Thus, as clearly shown by the preceding examples, expression of and cell wall isolation of exogenous proteins in plant tissue can easily be facilitated by utilizing any of the cellulose binding peptides of the present invention.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A process of expressing a recombinant protein in a plant and of isolating the recombinant protein from the plant, the process comprising the steps of:

(a) providing a plant, a plant derived tissue or cultured plant cells expressing a fusion protein including the recombinant protein and a cellulose binding peptide being fused thereto, said fusion protein being compartmentalized within cells of said plant, plant derived tissue or cultured plant cells, so as to be sequestered from cell walls of said cells of said plant, plant derived tissue or cultured plant cells;

(b) homogenizing said plant, plant derived tissue or cultured plant cells, so as to bring into contact said fusion protein with a plant derived cellulosic matter of said plant, plant derived tissue or cultured plant cells, to thereby effect affinity binding of said fusion protein via said cellulose binding peptide to said cellulosic matter, thereby obtaining a fusion protein cellulosic matter complex; and

(c) isolating said fusion protein cellulosic matter complex.

2. The process of claim 1, further comprising the step of:

(d) washing said fusion protein cellulosic matter complex, thereby removing endogenous plant proteins and other plant material therefrom.

3. The process of claim 2, further comprising the step of:

(e) collecting said fusion protein cellulosic matter complex as a final product of the process.

4. The process of claim 2, further comprising the step of:

(e) exposing said fusion protein cellulosic matter complex to conditions effective in dissociating said fusion protein from said cellulosic matter; and

(f) isolating said fusion protein, thereby obtaining an isolated fusion protein.

5. The process of claim 4, wherein said conditions effective in dissociating said fusion protein from said cellulosic matter are selected from the group consisting of basic conditions, denaturative conditions and affinity displacement conditions.

6. The process of claim 4, further comprising the step of:

(g) exposing said isolated fusion protein to conditions effective in digesting said fusion protein so as to release said recombinant protein therefrom, thereby obtaining a released recombinant protein.

7. The process of claim 6, wherein said conditions effective in digesting said fusion protein so as to release said recombinant protein therefrom are selected from the group consisting of proteolysis effected via a protease and proteolysis effected under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

8. The process of claim 4, further comprising the step of:

(h) isolating said released recombinant protein.

9. The process of claim 2, further comprising the step of:

(e) exposing said fusion protein cellulosic matter complex to conditions effective in digesting said fusion protein so as to release said recombinant protein therefrom, thereby obtaining a released recombinant protein.

10. The process of claim 6, wherein said conditions effective in digesting said fusion protein so as to release said recombinant protein therefrom are selected from the group consisting of proteolysis effected via a protease and proteolysis effected under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

11. The process of claim 4, further comprising the step of:

(f) isolating said released recombinant protein.

12. A genetically modified or viral infected plant or cultured plant cells expressing a fusion protein including a recombinant protein and a cellulose binding peptide, said fusion protein being compartmentalized

within cells of said plant or cultured plant cells, so as to be sequestered from cell walls of said cells of said plant or cultured plant cells.

13. The genetically modified or viral infected plant or cultured plant cells of claim 12, wherein expression of said fusion protein is under a control of a constitutive or tissue specific plant promoter.

14. The genetically modified or viral infected plant or cultured plant cells of claim 12, wherein said fusion protein is compartmentalized within a cellular compartment selected from the group consisting of cytoplasm, endoplasmic reticulum, golgi apparatus, oil bodies, starch bodies, chloroplastids, chloroplasts, chromoplastids, chromoplasts, vacuole, lysosomes, mitochondria, and nucleus.

15. A genetically modified or viral infected plant or cultured plant cells expressing a fusion protein including a recombinant protein and a cellulose binding peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence.

16. The genetically modified or viral infected plant or cultured plant cells of claim 15, wherein said fusion protein is compartmentalized within cells of said plant or cultured plant cells, so as to be sequestered from cell walls of said cells of said plant or cultured plant cells.

17. The genetically modified or viral infected plant or cultured plant cells of claim 15, wherein said fusion protein is compartmentalized within a cellular compartment selected from the group consisting of cytoplasm, endoplasmatic rericulum, golgi apparatus, oil bodies, starch bodies, chloroplastids, chloroplasts, chromoplastids, chromoplasts, vacuole, lysosomes, mitochondria, and nucleus.

18. The genetically modified or viral infected plant or cultured plant cells of claim 15, wherein expression of said fusion protein is under a control of a constitutive or tissue specific plant promoter.

19. A composition of matter comprising:

(a) a plant derived cellulosic matter of a plant; and

(b) a fusion protein including a recombinant protein and a cellulose binding peptide separated therebetween via a unique amino acid sequence recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, said fusion protein being expressed in said plant and complexed to said plant derived cellulosic matter of said plant by affinity binding via said cellulose binding peptide.

20. A nucleic acid molecule comprising:

(a) a promoter sequence for directing protein expression in plant cells;

(b) a heterologous nucleic acid sequence including:

(i) a first sequence encoding a cellulose binding peptide;

(ii) a second sequence encoding a recombinant protein, wherein said first and second sequences are joined together in frame; and

(iii) a third sequence encoding a unique amino acid sequence being recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, said third sequence being between and in frame with said first and second sequences;

wherein, said heterologous nucleic acid sequence being down stream said promoter sequence, such that expression of said heterologous nucleic acid sequence is effectable by said promoter sequence.

21. The nucleic acid molecule of claim 20, further comprising a sequence element selected from the group consisting of an origin of replication for propagation in bacterial cells, at least one sequence element for integration into a plant's genome, a polyadenylation recognition sequence, a transcription - termination signal, a sequence encoding a translation start site, a sequence encoding a translation stop site, plant RNA virus derived sequences, plant DNA virus derived sequences, tumor inducing (Ti) plasmid derived sequences, and a transposable element derived sequence.

22. A nucleic acid molecule comprising:

(a) a promoter sequence for directing protein expression in plant cells;

(b) a heterologous nucleic acid sequence including:

(i) a first sequence encoding a cellulose binding peptide;

(ii) a second sequence encoding a recombinant protein, wherein said first and second sequences are joined together in frame; and

(iii) a third sequence encoding a signal peptide for directing a protein to a cellular compartment, said third sequence being upstream and in frame with said first and second sequences;

wherein, said heterologous nucleic acid sequence being down stream said promoter sequence, such that expression of said heterologous nucleic acid sequence is effectable by said promoter sequence.

23. The nucleic acid molecule of claim 22, wherein said heterologous nucleic acid sequence further includes

(iv) a fourth sequence encoding a unique amino acid sequence being recognizable and digestible by a protease or under predetermined cis or trans conditions for digesting a controllable intervening protein sequence, said fourth sequence being between and in frame with said first and second sequences.

24. The nucleic acid molecule of claim 22, further comprising a sequence element selected from the group consisting of an origin of replication for propagation in bacterial cells, at least one sequence element for integration into a plant's genome, a polyadenylation recognition sequence, a transcription termination signal, a sequence encoding a translation start site, a sequence encoding a translation stop site, plant RNA virus derived sequences, plant DNA virus derived sequences, tumor inducing (Ti) plasmid derived sequences, and a transposable element derived sequence.

25. The nucleic acid molecule of claim 22, wherein said cellular compartment is selected from the group consisting of cytoplasm, endoplasmic reticulum, golgi apparatus, oil bodies, starch bodies,

chloroplastids, chloroplasts, chromoplastids, chromoplasts, vacuole, lysosomes, mitochondria, and nucleus.

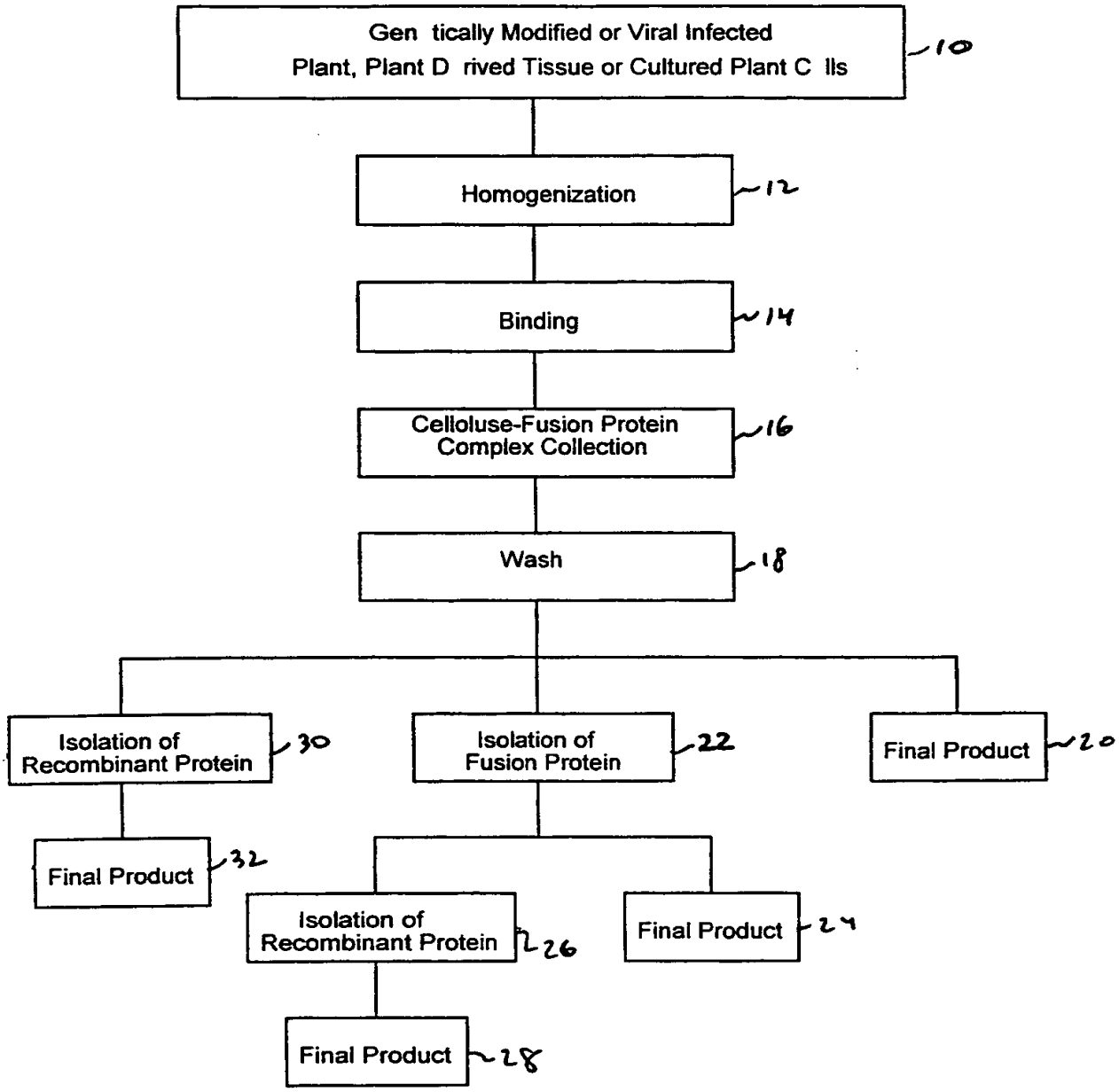
Genetically Modified or Viral Infected Plant, Plant Derived Tissue or Cultured Plant Cells
10
Homogenization
12
Binding
14
Celloluse-Fusion Protein Complex Collection
16
Wash
18
Isolation of Recombinant Protein
30
Isolation of Fusion Protein
22
Final Product
20
Final Product
32
Isolation of Recombinant Protein
26
Final Product
24
Final Product
28

FIG. 1

```
1       GGGGATCTATGGCGCGAAAATCCCTAATTTTCCCGGTGATTTTGCTCGCCGTTCTTCTCT
1                M  A  R  K  S  L  I  F  P  V  I  L  L  A  V  L  L

61      TCTCTCCGCCGATTTACTCCGCCGGTCACGATTACCGCGACGCTCTCCGTAAATCTAGAA
18      F  S  P  P  I  Y  S  A  G  H  D  Y  R  D  A  L  R  K  S  R

121     TGGCGGCGGTAGAAAATAAAGAAGAAACACCAGAAACACCAGAAACTGATTCAGAAGAAG
38      M  A  A  V  E  N  K  E  E  T  P  E  T  P  E  T  D  S  E  E

181     AAGTAACAATCAAAGCTAACCTAATCTTTGCAAATGGAAGCACACAAACTGCAGAATTCA
58      E  V  T  I  K  A  N  L  I  F  A  N  G  S  T  Q  T  A  E  F

241     AAGGAACATTTGAAAAAGCAACATCAGAAGCTTATGAGTATGCAGATACTTTGAAGAAAG
78      K  G  T  F  E  K  A  T  S  E  A  Y  E  Y  A  D  T  L  K  K

301     ACAATGGAGAATATACTGTAGATGTTGCAGATAAAGGTTATACTTTAAATATTAAATTTG
98      D  N  G  E  Y  T  V  D  V  A  D  K  G  Y  T  L  N  I  K  F

361     CTGGAAAAGAAAAAACACCAGAAGAACCAAAAGAAGAAGTTACTATTAAAGCAAACTTAA
118     A  G  K  E  K  T  P  E  E  P  K  E  E  V  T  I  K  A  N  L

421     TCTATGCAGATGGAAAAACACAAACAGCAGAATTCAAAGGAACATTTGAAGAAGCAACAG
138     I  Y  A  D  G  K  T  Q  T  A  E  F  K  G  T  F  E  E  A  T

481     CAGAAGCATACAGATATGCAGATGCATTAAAGAAGGACAATGGAGAATATACAGTAGACG
158     A  E  A  Y  R  Y  A  D  A  L  K  K  D  N  G  E  Y  T  V  D

541     TTGCAGATAAAGGTTATACTTTAAATATTAAATTTGCTGGAAAAGAAAAAACACCAGAAG
178     V  A  D  K  G  Y  T  L  N  I  K  F  A  G  K  E  K  T  P  E

601     AACCAAAAGAAGAAGTTACTATTAAAGCAAACTTAATCTATGCAGATGGAAAAACACAAA
198     E  P  K  E  E  V  T  I  K  A  N  L  I  Y  A  D  G  K  T  Q

661     CAGCAGAATTCAAAGGAACATTTGAAGAAGCAACAGCAGAAGCATACAGATATGCTGACT
218     T  A  E  F  K  G  T  F  E  E  A  T  A  E  A  Y  R  Y  A  D

721     TATTAGCAGCAAAAGAAAATGGTAAATATACAGTAGACGTTGCAGATAAAGGTTATACTT
238     L  L  A  A  K  E  N  G  K  Y  T  V  D  V  A  D  K  G  Y  T

781     TAAATATTAAATTTGCTGGAAAAGAAAAAACACCAGAAGAACCAAAAGAAGAAGTTACTA
258     L  N  I  K  F  A  G  K  E  K  T  P  E  E  P  K  E  E  V  T

841     TTAAAGCAAACTTAATCTATGCAGATGGAAAAACTCAAACAGCAGAGTTCAAAGGAACAT
278     I  K  A  N  L  I  Y  A  D  G  K  T  Q  T  A  E  F  K  G  T

901     TTGCAGAAGCAACAGCAGAAGCATACAGATACGCTGACTTATTAGCAAAAGAAAATGGTA
298     F  A  E  A  T  A  E  A  Y  R  Y  A  D  L  L  A  K  E  N  G

961     AATATACAGCAGACTTAGAAGATGGTGGATACACTATTAATATTAGATTTGCAGGTAAGA
318     K  Y  T  A  D  L  E  D  G  G  Y  T  I  N  I  R  F  A  G  K

1021    AAGTTGACGAAAAACCAGAAGGGATCCCTCCGACGCCGACCCCGACTAGTGCTAGCGGTC
338     K  V  D  E  K  P  E  G  I  P  P  T  P  T  P  T  S  A  S  G
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Fig. 2a

FIG. 2a CONT.

```
1081    CAGCCGGCTGCCAGGTTCTGTGGGGTGTTAACCAGTGGAACACCGGTTTCACCGCTCAGG
358     P  A  G  C  Q  V  L  W  G  V  N  Q  W  N  T  G  F  T  A  Q

1141    TTACCGTTAAAAACACGGGCTCAGCTCCGGTTGACGGTTGGACCCTGACCTTCTCTTTTC
378     V  T  V  K  N  T  G  S  A  P  V  D  G  W  T  L  T  F  S  F

1201    CCTCGGGTCAGCAGGTAACTCAGGCTTGGTCATCTACAGTTACCCAGTCTGGATCCGCTG
398     P  S  G  Q  Q  V  T  Q  A  W  S  S  T  V  T  Q  S  G  S  A

1261    TTACAGTTCGTAACGCTCCGTGGAACGGTAATATTCCTGCAGGTGGAACCGCTCAGTTCG
418     V  T  V  R  N  A  P  W  N  G  N  I  P  A  G  G  T  A  Q  F

1321    GTTTCCAAGGTTCTCACACCGGTACCAACGCGGCGCCAACCGCTTTCTCTCTGAACGGGG
438     G  F  Q  G  S  H  T  G  T  N  A  A  P  T  A  F  S  L  N  G

1381    CCCCTTGCACCGTTGGACATGATGAACTTTAAGTCGAC
458     A  P  C  T  V  G  H  D  E  L
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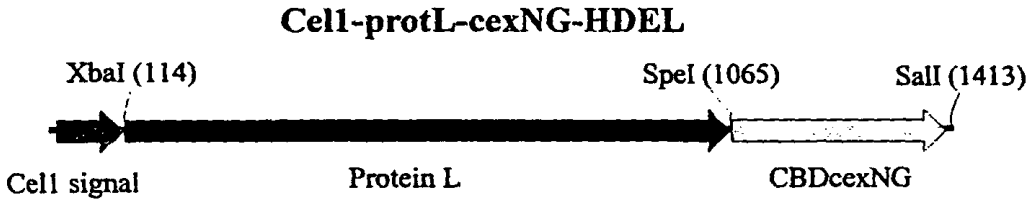

FIG. 2b

```
61      GCCCCCCCCTCGAGCCCGGGATGGCGCGAAAATCCCTAATTTTCCCGGTGATTTTGCTCGC
1                              M  A  R  K  S  L  I  F  P  V  I  L  L  A

121     CGTTCTTCTCTTCTCTCCGCCGATTTACTCCGCCGGTCACGATTACCGCGACGCTCTCCG
15        V  L  L  F  S  P  P  I  Y  S  A  G  H  D  Y  R  D  A  L  R

181     TAAGACGTCCGGTCCAGCCGGCTGCCAGGTTCTGTGGGGTGTTAACCAGTGGAACACCGG
35        K  T  S  G  P  A  G  C  Q  V  L  W  G  V  N  Q  W  N  T  G

241     TTTCACCGCTCAGGTTACCGTTAAAAACACGGGCTCAGCTCCGGTTGACGGTTGGACCCT
55        F  T  A  Q  V  T  V  K  N  T  G  S  A  P  V  D  G  W  T  L

301     GACCTTCTCTTTTCCCTCGGGTCAGCAGGTAACTCAGGCTTGGTCATCTACAGTTACCCA
75        T  F  S  F  P  S  G  Q  Q  V  T  Q  A  W  S  S  T  V  T  Q

361     GTCTGGATCCGCTGTTACAGTTCGTAACGCTCCGTGGAACGGTAATATTCCTGCAGGTGG
95        S  G  S  A  V  T  V  R  N  A  P  W  N  G  N  I  P  A  G  G

421     AACCGCTCAGTTCGGTTTCCAAGGTTCTCACACCGGTACCAACGCGGCGCCAACCGCTTT
115       T  A  Q  F  G  F  Q  G  S  H  T  G  T  N  A  A  P  T  A  F

481     CTCTCTGAACGGGGCCCCTTGCACCGTTGGTCCGACTACCTCACCTACAACGCGTAAGCT
135       S  L  N  G  A  P  C  T  V  G  P  T  T  S  P  T  T  R  K  L

541     CTGCAGCCTGGACAACGGGGACTGTGACCAGTTCTGCCACGAGGAACAGAACTCTGTGGT
155       C  S  L  D  N  G  D  C  D  Q  F  C  H  E  E  Q  N  S  V  V

601     GTGCTCCTGCGCCCGCGGGTACACCCTGGCTGACAACGGCAAGGCCTGCATTCCCACAGG
175       C  S  C  A  R  G  Y  T  L  A  D  N  G  K  A  C  I  P  T  G

661     GCCCTACCCCTGTGGGAAACAGACCCTGGAACGCAGGAAGAGGTCAGTGGCCCAGGCCAC
195       P  Y  P  C  G  K  Q  T  L  E  R  R  K  R  S  V  A  Q  A  T

721     CAGCAGCAGCGGGGAGGCCCCTGACAGCATCACATGGAAGCCATATGATGCAGCCGACCT
215       S  S  S  G  E  A  P  D  S  I  T  W  K  P  Y  D  A  A  D  L

781     GGACCCCACCGAGAACCCCTTCGACCTGCTTGACTTTGATCAGACGCAGCCTGAGAGGGG
235       D  P  T  E  N  P  F  D  L  L  D  F  D  Q  T  Q  P  E  R  G

841     CGACAACAACATTGAAGGTCGTATCGTGGGAGGCCAGGAATGCAAGGACGGGGAGTGTCC
255       D  N  N  I  E  G  R  I  V  G  G  Q  E  C  K  D  G  E  C  P

901     CTGGCAGGCCCTGCTCATCAATGAGGAAAACGAGGGTTTCTGTGGTGGAACCATTCTGAG
275       W  Q  A  L  L  I  N  E  E  N  E  G  F  C  G  G  T  I  L  S

961     CGAGTTCTACATCCTAACGGCAGCCCACTGTCTCTACCAAGCCAAGAGATTCAAGGTGAG
295       E  F  Y  I  L  T  A  A  H  C  L  Y  Q  A  K  R  F  K  V  R

1021    GGTAGGGGACCGGAACACGGAGCAGGAGGAGGGCGGTGAGGCGGTGCACGAGGTGGAGGT
315       V  G  D  R  N  T  E  Q  E  E  G  G  E  A  V  H  E  V  E  V

1081    GGTCATCAAGCACAACCGGTTCACAAAGGAGACCTATGACTTCGACATCGCCGTGCTCCG
335       V  I  K  H  N  R  F  T  K  E  T  Y  D  F  D  I  A  V  L  R

1141    GCTCAAGACCCCCATCACCTTCCGCATGAACGTGGCGCCTGCCTGCCTCCCCGAGCGTGA
355       L  K  T  P  I  T  F  R  M  N  V  A  P  A  C  L  P  E  R  D
```

FIG. 3a

FIG. 3a CONT.

```
1201    CTGGGCCGAGTCCACGCTGATGACGCAGAAGACGGGGATTGTGAGCGGCTTCGGGCGCAC
375       W  A  E  S  T  L  M  T  Q  K  T  G  I  V  S  G  F  G  R  T

1261    CCACGAGAAGGGCCGGCAGTCCACCAGGCTCAAGATGCTGGAGGTGCCCTACGTGGACCG
395       H  E  K  G  R  Q  S  T  R  L  K  M  L  E  V  P  Y  V  D  R

1321    CAACAGCTGCAAGCTGTCCAGCAGCTTCATCATCACCCAGAACATGTTCTGTGCCGGCTA
415       N  S  C  K  L  S  S  S  F  I  I  T  Q  N  M  F  C  A  G  Y

1381    CGACACCAAGCAGGAGGATGCCTGCCAGGGGGACAGCGGGGGCCCGCACGTCACCCGCTT
435       D  T  K  Q  E  D  A  C  Q  G  D  S  G  G  P  H  V  T  R  F

1441    CAAGGACACCTACTTCGTGACAGGCATCGTCAGCTGGGGAGAGGGCTGTGCCCGTAAGGG
455       K  D  T  Y  F  V  T  G  I  V  S  W  G  E  G  C  A  R  K  G

1501    GAAGTACGGGATCTACACCAAGGTCACCGCCTTCCTCAAGTGGATCGACAGGTCCATGAA
475       K  Y  G  I  Y  T  K  V  T  A  F  L  K  W  I  D  R  S  M  K

1561    AACCAGGGGCTTGCCCAAGGCCAAGCCTACTAGTCATGATGAACTTTAAGAGCTCCAGCT
495       T  R  G  L  P  K  A  K  P  T  S  H  D  E  L
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Sig-cex-Fx-HDEL

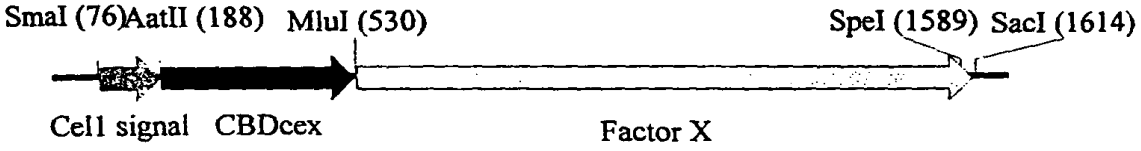

FIG. 3b

```
61      GCCCCCCCCTCGAGCCCGGGATGGCGCGAAAATCCCTAATTTTCCCGGTGATTTTGCTCGC
1                              M  A  R  K  S  L  I  F  P  V  I  L  L  A

121     CGTTCTTCTCTTCTCTCCGCCGATTTACTCCGCCGGTCACGATTACCGCGACGCTCTCCG
15       V  L  L  F  S  P  P  I  Y  S  A  G  H  D  Y  R  D  A  L  R

181     TAAGACGTCGGCTAGCGGAATAATGGTAGCGACAGCAAAATACGGAACACCGGTCATCGA
35       K  T  S  A  S  G  I  M  V  A  T  A  K  Y  G  T  P  V  I  D

241     TGGAGAGATAGACGAGATCTGGAACACGACAGAGGAGATAGAGACGAAAGCGGTGGCCAT
55       G  E  I  D  E  I  W  N  T  T  E  E  I  E  T  K  A  V  A  M

301     GGGATCGCTTGACAAGAACGCGACAGCGAAAGTGAGGGTGCTGTGGGACGAGAACTACCT
75       G  S  L  D  K  N  A  T  A  K  V  R  V  L  W  D  E  N  Y  L

361     GTACGTACTTGCAATCGTGAAAGACCCCGTTCTGAACAAAGACAACAGCAACCCGTGGGA
95       Y  V  L  A  I  V  K  D  P  V  L  N  K  D  N  S  N  P  W  E

421     ACAGGATTCCGTGGAGATCTTCATCGACGAGAACAACCACAAGACAGGATACTACGAAGA
115      Q  D  S  V  E  I  F  I  D  E  N  N  H  K  T  G  Y  Y  E  D

481     CGACGACGCACAGTTCAGGGTGAACTACATGAACGAGCAGACGTTTGGAACGGGAGGAAG
135      D  D  A  Q  F  R  V  N  Y  M  N  E  Q  T  F  G  T  G  G  S

541     TCCAGCGAGGTTCAAGACAGCGGTGAAACTGATCGAAGGAGGATACATAGTTGAGGCAGC
155      P  A  R  F  K  T  A  V  K  L  I  E  G  G  Y  I  V  E  A  A

601     GATCAAGTGGAAGACGATCAAACCCACACCGAACACGGTGATAGGATTCAACATCCAGGT
175      I  K  W  K  T  I  K  P  T  P  N  T  V  I  G  F  N  I  Q  V

661     GAACGATGCGAACGAGAAAGGGCAGAGGGTCGGTATCATCTCCTGGAGCGATCCCACAAA
195      N  D  A  N  E  K  G  Q  R  V  G  I  I  S  W  S  D  P  T  N

721     CAACAGCTGGAGAGATCCTTCAAAGTTCGGTAACCTCAGGCTCATCAAGGGATCTGGTCC
215      N  S  W  R  D  P  S  K  F  G  N  L  R  L  I  K  G  S  G  P

781     GACCCCATCCCCAACGCGTAAGCTCTGCAGCCTGGACAACGGGGACTGTGACCAGTTCTG
235      T  P  S  P  T  R  K  L  C  S  L  D  N  G  D  C  D  Q  F  C

841     CCACGAGGAACAGAACTCTGTGGTGTGCTCCTGCGCCCGCGGGTACACCCTGGCTGACAA
255      H  E  E  Q  N  S  V  V  C  S  C  A  R  G  Y  T  L  A  D  N

901     CGGCAAGGCCTGCATTCCCACAGGGCCCTACCCCTGTGGGAAACAGACCCTGGAACGCAG
275      G  K  A  C  I  P  T  G  P  Y  P  C  G  K  Q  T  L  E  R  R

961     GAAGAGGTCAGTGGCCCAGGCCACCAGCAGCAGCGGGGAGGCCCCTGACAGCATCACATG
295      K  R  S  V  A  Q  A  T  S  S  S  G  E  A  P  D  S  I  T  W

1021    GAAGCCATATGATGCAGCCGACCTGGACCCCACCGAGAACCCCTTCGACCTGCTTGACTT
315      K  P  Y  D  A  A  D  L  D  P  T  E  N  P  F  D  L  L  D  F

1081    TGATCAGACGCAGCCTGAGAGGGGCGACAACAACATTGAAGGTCGTATCGTGGGAGGCCA
335      D  Q  T  Q  P  E  R  G  D  N  N  I  E  G  R  I  V  G  G  Q

1141    GGAATGCAAGGACGGGGAGTGTCCCTGGCAGGCCCTGCTCATCAATGAGGAAAACGAGGG
355      E  C  K  D  G  E  C  P  W  Q  A  L  L  I  N  E  E  N  E  G
```

Fig. 4a

Fig. 4a cont.

```
1201    TTTCTGTGGTGGAACCATTCTGAGCGAGTTCTACATCCTAACGGCAGCCCACTGTCTCTA
375       F  C  G  G  T  I  L  S  E  F  Y  I  L  T  A  A  H  C  L  Y

1261    CCAAGCCAAGAGATTCAAGGTGAGGGTAGGGGACCGGAACACGGAGCAGGAGGAGGGCGG
395       Q  A  K  R  F  K  V  R  V  G  D  R  N  T  E  Q  E  E  G  G

1321    TGAGGCGGTGCACGAGGTGGAGGTGGTCATCAAGCACAACCGGTTCACAAAGGAGACCTA
415       E  A  V  H  E  V  E  V  V  I  K  H  N  R  F  T  K  E  T  Y

1381    TGACTTCGACATCGCCGTGCTCCGGCTCAAGACCCCCATCACCTTCCGCATGAACGTGGC
435       D  F  D  I  A  V  L  R  L  K  T  P  I  T  F  R  M  N  V  A

1441    GCCTGCCTGCCTCCCCGAGCGTGACTGGGCCGAGTCCACGCTGATGACGCAGAAGACGGG
455       P  A  C  L  P  E  R  D  W  A  E  S  T  L  M  T  Q  K  T  G

1501    GATTGTGAGCGGCTTCGGGCGCACCCACGAGAAGGGCCGGCAGTCCACCAGGCTCAAGAT
475       I  V  S  G  F  G  R  T  H  E  K  G  R  Q  S  T  R  L  K  M

1561    GCTGGAGGTGCCCTACGTGGACCGCAACAGCTGCAAGCTGTCCAGCAGCTTCATCATCAC
495       L  E  V  P  Y  V  D  R  N  S  C  K  L  S  S  S  F  I  I  T

1621    CCAGAACATGTTCTGTGCCGGCTACGACACCAAGCAGGAGGATGCCTGCCAGGGGGACAG
515       Q  N  M  F  C  A  G  Y  D  T  K  Q  E  D  A  C  Q  G  D  S

1681    CGGGGGCCCGCACGTCACCCGCTTCAAGGACACCTACTTCGTGACAGGCATCGTCAGCTG
535       G  G  P  H  V  T  R  F  K  D  T  Y  F  V  T  G  I  V  S  W

1741    GGGAGAGGGCTGTGCCCGTAAGGGGAAGTACGGGATCTACACCAAGGTCACCGCCTTCCT
555       G  E  G  C  A  R  K  G  K  Y  G  I  Y  T  K  V  T  A  F  L

1801    CAAGTGGATCGACAGGTCCATGAAAACCAGGGGCTTGCCCAAGGCCAAGCCTACTAGTCA
575       K  W  I  D  R  S  M  K  T  R  G  L  P  K  A  K  P  T  S  H

1861    TGATGAACTTTAAGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGG
595       D  E  L
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Cel1-TmaIX-Fx-HDEL

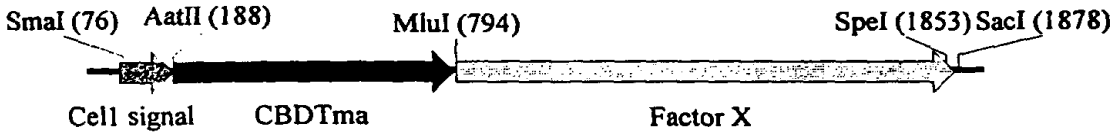

Fig. 4b


Plant material
Homogenize
Bind to
Centrifuge
Pellet, cell wall material (including cellulose)
Supernatant
Wash 3 times
Add Avicel (2%)
Analyze SDS-PAGE
Bind to
Wash
Analyze SDS-PAGE

Fig. 5

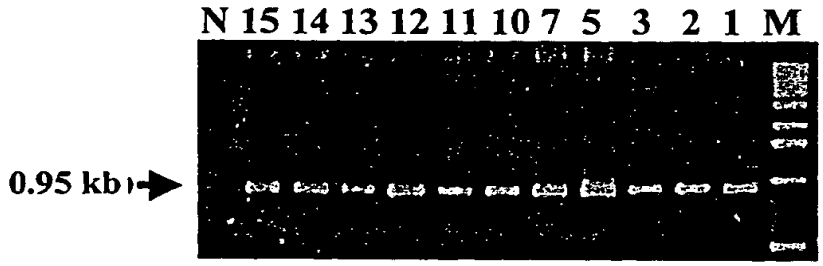
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Fig. 6

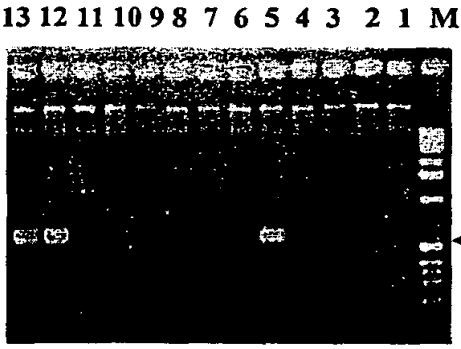

Fig. 7a

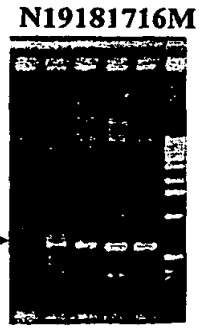

Fig. 7b

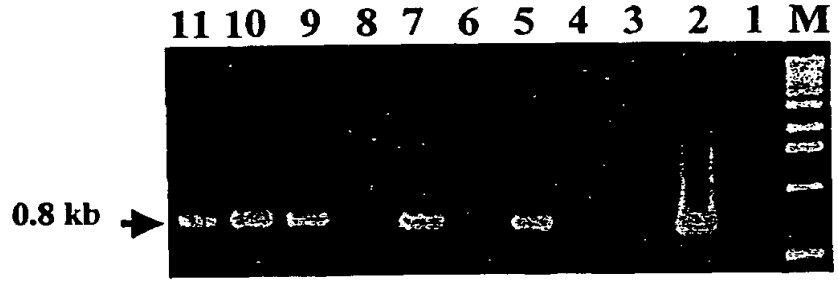

Fig. 8a

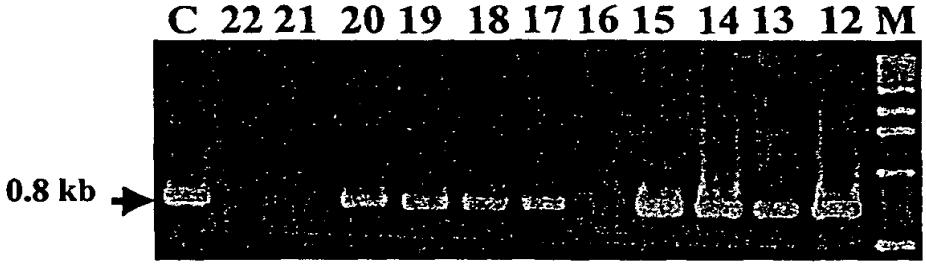

Fig. 8b

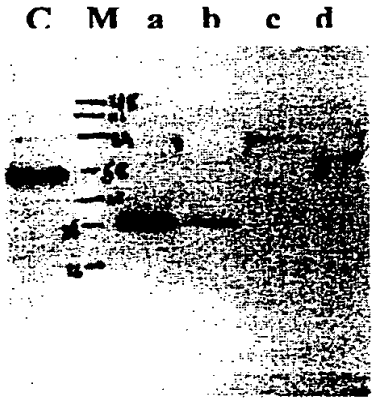

Fig. 9a

ProtL-cex

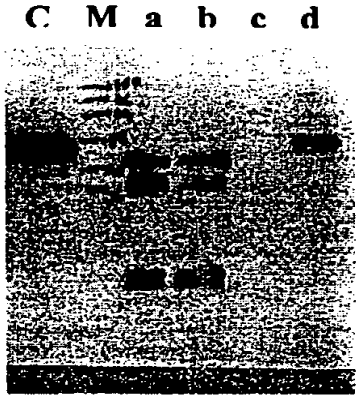

Fig. 9b

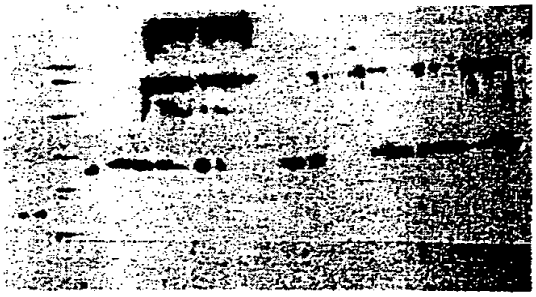

Fig. 10a

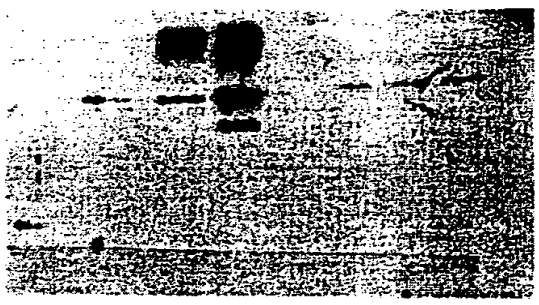

Fig. 10b

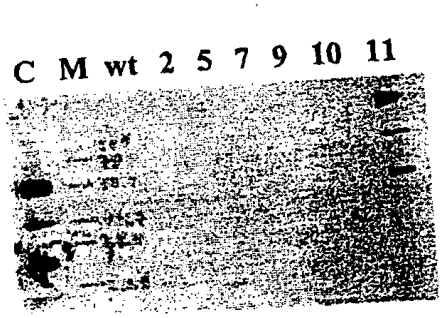

FIG. 11a

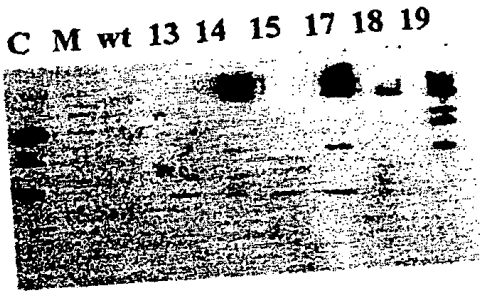

FIG. 11c

FIG. 11b

FIG. 11d

SEQUENCE LISTING

<110> Ziv Shani *et al.*

<120> PROCESS OF EXPRESSING AND ISOLATING RECOMBINANT PROTEINS AND RECOMBINANT PROTEIN PRODUCTS FROM PLANTS, PLANT DERIVED TISSUES OR CULTURED PLANT CELLS

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<150> 09/329,234

<151> June 10, 1999

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ACTTATTAGC AGCAAAAGAA AATGGTAAAT ATACAGTAGA CGTTGCAGAT AAAGGTTATA 660

CTTTAAATAT TAAATTTGCT GGAAAAGAAA AAACACCAGA AGAACCAAAA GAAGAAGTTA 720

CTATTAAAGC AAACTTAATC TATGCAGATG GAAAAACTCA AACAGCAGAG TTCAAAGGAA 780

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GTAAATATAC AGCAGACTTA GAAGATGGTG GATACACTAT TAATATTAGA TTTGCAGGTA 900

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CCGTGAAGAA CACGTCCTCC GCTCCGGTAG ACGGCTGGAC GCTCACGTTC AGCTTCCCGT 1080

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TGGCGGCGGT AGAAAATAAA GAAGAAACAC CAGAAACACC AGAAACTGAT TCAGAAGAAG 180

AAGTAACAAT CAAAGCTAAC CTAATCTTTG CAAATGGAAG CACACAAACT GCAGAATTCA 240

AAGGAACATT TGAAAAAGCA ACATCAGAAG CTTATGAGTA TGCAGATACT TTGAAGAAAG 300

ACAATGGAGA ATATACTGTA GATGTTGCAG ATAAAGGTTA TACTTTAAAT ATTAAATTTG 360

CTGGAAAAGA AAAAACACCA GAAGAACCAA AAGAAGAAGT TACTATTAAA GCAAACTTAA 420

TCTATGCAGA TGGAAAAACA CAAACAGCAG AATTCAAAGG AACATTTGAA GAAGCAACAG 480

CAGAAGCATA CAGATATGCA GATGCATTAA AGAAGGACAA TGGAGAATAT ACAGTAGACG 540

TTGCAGATAA AGGTTATACT TTAAATATTA AATTTGCTGG AAAAGAAAAA ACACCAGAAG 600

AACCAAAAGA AGAAGTTACT ATTAAAGCAA ACTTAATCTA TGCAGATGGA AAAACACAAA 660

CAGCAGAATT CAAAGGAACA TTTGAAGAAG CAACAGCAGA AGCATACAGA TATGCTGACT 720

TATTAGCAGC AAAAGAAAAT GGTAAATATA CAGTAGACGT TGCAGATAAA GGTTATACTT 780

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            20                  25                  30

Leu Arg Lys Ser Arg Met Ala Ala Val Glu Asn Lys Glu Glu Thr Pro
        35                  40                  45

Glu Thr Pro Glu Thr Asp Ser Glu Glu Glu Val Thr Ile Lys Ala Asn
    50                  55                  60

Leu Ile Phe Ala Asn Gly Ser Thr Gln Thr Ala Glu Phe Lys Gly Thr
65                  70                  75                  80

Phe Glu Lys Ala Thr Ser Glu Ala Tyr Glu Tyr Ala Asp Thr Leu Lys
                85                  90                  95

Lys Asp Asn Gly Glu Tyr Thr Val Asp Val Ala Asp Lys Gly Tyr Thr
            100                 105                 110

Leu Asn Ile Lys Phe Ala Gly Lys Glu Lys Thr Pro Glu Glu Pro Lys
        115                 120                 125

Glu Glu Val Thr Ile Lys Ala Asn Leu Ile Tyr Ala Asp Gly Lys Thr
    130                 135                 140

Gln Thr Ala Glu Phe Lys Gly Thr Phe Glu Glu Ala Thr Ala Glu Ala
145                 150                 155                 160

Tyr Arg Tyr Ala Asp Ala Leu Lys Lys Asp Asn Gly Glu Tyr Thr Val
                165                 170                 175

Asp Val Ala Asp Lys Gly Tyr Thr Leu Asn Ile Lys Phe Ala Gly Lys
            180                 185                 190

Glu Lys Thr Pro Glu Glu Pro Lys Glu Glu Val Thr Ile Lys Ala Asn
        195                 200                 205

Leu Ile Tyr Ala Asp Gly Lys Thr Gln Thr Ala Glu Phe Lys Gly Thr
    210                 215                 220

Phe Glu Glu Ala Thr Ala Glu Ala Tyr Arg Tyr Ala Asp Leu Leu Ala
225                 230                 235                 240

Ala Lys Glu Asn Gly Lys Tyr Thr Val Asp Val Ala Asp Lys Gly Tyr
                245                 250                 255

Thr Leu Asn Ile Lys Phe Ala Gly Lys Glu Lys Thr Pro Glu Glu Pro
            260                 265                 270

Lys Glu Glu Val Thr Ile Lys Ala Asn Leu Ile Tyr Ala Asp Gly Lys
        275                 280                 285

Thr Gln Thr Ala Glu Phe Lys Gly Thr Phe Ala Glu Ala Thr Ala Glu
    290                 295                 300

Ala Tyr Arg Tyr Ala Asp Leu Leu Ala Lys Glu Asn Gly Lys Tyr Thr
305                 310                 315                 320

Ala Asp Leu Glu Asp Gly Gly Tyr Thr Ile Asn Ile Arg Phe Ala Gly
                325                 330                 335

Lys Lys Val Asp Glu Lys Pro Glu Gly Ile Pro Pro Thr Pro Thr Pro
            340                 345                 350

Thr Ser Ala Ser Gly Pro Ala Gly Cys Gln Val Leu Trp Gly Val Asn
        355                 360                 365

Gln Trp Asn Thr Gly Phe Thr Ala Gln Val Thr Val Lys Asn Thr Gly
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370 375 380

Ser Ala Pro Val Asp Gly Trp Thr Leu Thr Phe Ser Phe Pro Ser Gly
385 390 395 400

Gln Gln Val Thr Gln Ala Trp Ser Ser Thr Val Thr Gln Ser Gly Ser
405 410 415

Ala Val Thr Val Arg Asn Ala Pro Trp Asn Gly Asn Ile Pro Ala Gly
420 425 430

Gly Thr Ala Gln Phe Gly Phe Gln Gly Ser His Thr Gly Thr Asn Ala
435 440 445

Ala Pro Thr Ala Phe Ser Leu Asn Gly Ala Pro Cys Thr Val Gly His
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Asp Glu Leu

465

<210> 13

<211> 25

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

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<210> 14

<211> 17

<212> DNA

<213> artificial sequence

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<223> Description of Artificial Sequence:PCR primer

<400> 14

CTTAAAGTTC ATCATGA 17

<210> 15

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 24

AAAACCCGGG ATGGCGCGAA AATC 24

<210> 16

<211> 33

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 16

AAAAGACGTC TTACGGAGAG CGTCGCGGTA ATC 33

<210> 17

<211> 1560

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:construct

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CGTTCTTCTC TTCTCTCCGC CGATTTACTC CGCCGGTCAC GATTACCGCG ACGCTCTCCG 120

TAAGACGTCC GGTCCAGCCG GCTGCCAGGT TCTGTGGGGT GTTAACCAGT GGAACACCGG 180

TTTCACCGCT CAGGTTACCG TTAAAAACAC GGGCTCAGCT CCGGTTGACG GTTGGACCCT 240

GACCTTCTCT TTTCCCTCGG GTCAGCAGGT AACTCAGGCT TGGTCATCTA CAGTTACCCA 300

GTCTGGATCC GCTGTTACAG TTCGTAACGC TCCGTGGAAC GGTAATATTC CTGCAGGTGG 360

AACCGCTCAG TTCGGTTTCC AAGGTTCTCA CACCGGTACC AACGCGGCGC CAACCGCTTT 420

CTCTCTGAAC GGGGCCCCTT GCACCGTTGG TCCGACTACC TCACCTACAA CGCGTAAGCT 480

CTGCAGCCTG GACAACGGGG ACTGTGACCA GTTCTGCCAC GAGGAACAGA ACTCTGTGGT 540

GTGCTCCTGC GCCCGCGGGT ACACCCTGGC TGACAACGGC AAGGCCTGCA TTCCCACAGG 600

GCCCTACCCC TGTGGGAAAC AGACCCTGGA ACGCAGGAAG AGGTCAGTGG CCCAGGCCAC 660

CAGCAGCAGC GGGGAGGCCC CTGACAGCAT CACATGGAAG CCATATGATG CAGCCGACCT 720

GGACCCCACC GAGAACCCCT TCGACCTGCT TGACTTTGAT CAGACGCAGC CTGAGAGGGG 780

CGACAACAAC ATTGAAGGTC GTATCGTGGG AGGCCAGGAA TGCAAGGACG GGGAGTGTCC 840

CTGGCAGGCC CTGCTCATCA ATGAGGAAAA CGAGGGTTTC TGTGGTGGAA CCATTCTGAG 900

CGAGTTCTAC ATCCTAACGG CAGCCCACTG TCTCTACCAA GCCAAGAGAT TCAAGGTGAG 960

GGTAGGGGAC CGGAACACGG AGCAGGAGGA GGGCGGTGAG GCGGTGCACG AGGTGGAGGT 1020

GGTCATCAAG CACAACCGGT TCACAAAGGA GACCTATGAC TTCGACATCG CCGTGCTCCG 1080

GCTCAAGACC CCCATCACCT TCCGCATGAA CGTGGCGCCT GCCTGCCTCC CCGAGCGTGA 1140

CTGGGCCGAG TCCACGCTGA TGACGCAGAA GACGGGGATT GTGAGCGGCT TCGGGCGCAC 1200

CCACGAGAAG GGCCGGCAGT CCACCAGGCT CAAGATGCTG GAGGTGCCCT ACGTGGACCG 1260

CAACAGCTGC AAGCTGTCCA GCAGCTTCAT CATCACCCAG AACATGTTCT GTGCCGGCTA 1320

CGACACCAAG CAGGAGGATG CCTGCCAGGG GGACAGCGGG GGCCCGCACG TCACCCGCTT 1380

CAAGGACACC TACTTCGTGA CAGGCATCGT CAGCTGGGGA GAGGGCTGTG CCCGTAAGGG 1440

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AACCAGGGGC TTGCCCAAGG CCAAGCCTAC TAGTCATGAT GAACTTTAAG AGCTCCAGCT 1560

<210> 18

<211> 509

<212> PRT

<213> Artificial sequence

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  1               5                  10                  15

Leu Phe Ser Pro Pro Ile Tyr Ser Ala Gly His Asp Tyr Arg Asp Ala
            20                  25                  30

Leu Arg Lys Thr Ser Gly Pro Ala Gly Cys Gln Val Leu Trp Gly Val
        35                  40                  45

Asn Gln Trp Asn Thr Gly Phe Thr Ala Gln Val Thr Val Lys Asn Thr
    50                  55                  60

Gly Ser Ala Pro Val Asp Gly Trp Thr Leu Thr Phe Ser Phe Pro Ser
65                  70                  75                  80

Gly Gln Gln Val Thr Gln Ala Trp Ser Ser Thr Val Thr Gln Ser Gly
                85                  90                  95

Ser Ala Val Thr Val Arg Asn Ala Pro Trp Asn Gly Asn Ile Pro Ala
            100                 105                 110

Gly Gly Thr Ala Gln Phe Gly Phe Gln Gly Ser His Thr Gly Thr Asn
        115                 120                 125

Ala Ala Pro Thr Ala Phe Ser Leu Asn Gly Ala Pro Cys Thr Val Gly
    130                 135                 140

Pro Thr Thr Ser Pro Thr Thr Arg Lys Leu Cys Ser Leu Asp Asn Gly
145                 150                 155                 160

Asp Cys Asp Gln Phe Cys His Glu Glu Gln Asn Ser Val Val Cys Ser
                165                 170                 175

Cys Ala Arg Gly Tyr Thr Leu Ala Asp Asn Gly Lys Ala Cys Ile Pro
            180                 185                 190

Thr Gly Pro Tyr Pro Cys Gly Lys Gln Thr Leu Glu Arg Arg Lys Arg
        195                 200                 205

Ser Val Ala Gln Ala Thr Ser Ser Ser Gly Glu Ala Pro Asp Ser Ile
    210                 215                 220

Thr Trp Lys Pro Tyr Asp Ala Ala Asp Leu Asp Pro Thr Glu Asn Pro
225                 230                 235                 240

Phe Asp Leu Leu Asp Phe Asp Gln Thr Gln Pro Glu Arg Gly Asp Asn
                245                 250                 255

Asn Ile Glu Gly Arg Ile Val Gly Gly Gln Glu Cys Lys Asp Gly Glu
            260                 265                 270
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Cys Pro Trp Gln Ala Leu Leu Ile Asn Glu Glu Asn Glu Gly Phe Cys
        275                 280                 285

Gly Gly Thr Ile Leu Ser Glu Phe Tyr Ile Leu Thr Ala Ala His Cys
    290                 295                 300

Leu Tyr Gln Ala Lys Arg Phe Lys Val Arg Val Gly Asp Arg Asn Thr
305                 310                 315                 320

Glu Gln Glu Glu Gly Gly Glu Ala Val His Glu Val Glu Val Val Ile
                325                 330                 335

Lys His Asn Arg Phe Thr Lys Glu Thr Tyr Asp Phe Asp Ile Ala Val
            340                 345                 350

Leu Arg Leu Lys Thr Pro Ile Thr Phe Arg Met Asn Val Ala Pro Ala
        355                 360                 365

Cys Leu Pro Glu Arg Asp Trp Ala Glu Ser Thr Leu Met Thr Gln Lys
    370                 375                 380

Thr Gly Ile Val Ser Gly Phe Gly Arg Thr His Glu Lys Gly Arg Gln
385                 390                 395                 400

Ser Thr Arg Leu Lys Met Leu Glu Val Pro Tyr Val Asp Arg Asn Ser
                405                 410                 415

Cys Lys Leu Ser Ser Ser Phe Ile Ile Thr Gln Asn Met Phe Cys Ala
            420                 425                 430

Gly Tyr Asp Thr Lys Gln Glu Asp Ala Cys Gln Gly Asp Ser Gly Gly
        435                 440                 445

Pro His Val Thr Arg Phe Lys Asp Thr Tyr Phe Val Thr Gly Ile Val
    450                 455                 460

Ser Trp Gly Glu Gly Cys Ala Arg Lys Gly Lys Tyr Gly Ile Tyr Thr
465                 470                 475                 480

Lys Val Thr Ala Phe Leu Lys Trp Ile Asp Arg Ser Met Lys Thr Arg
                485                 490                 495

Gly Leu Pro Lys Ala Lys Pro Thr Ser His Asp Glu Leu
            500                 505
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<211> 32

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 19

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<210> 20

<211> 27

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 20

AAAAACGCGT TGGGGATGGG GTCGGAC 27

<210> 21

<211> 1860

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:construct

<400> 21

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TAAGACGTCG GCTAGCGGAA TAATGGTAGC GACAGCAAAA TACGGAACAC CGGTCATCGA 180

TGGAGAGATA GACGAGATCT GGAACACGAC AGAGGAGATA GAGACGAAAG CGGTGGCCAT 240

GGGATCGCTT GACAAGAACG CGACAGCGAA AGTGAGGGTG CTGTGGGACG AGAACTACCT 300

GTACGTACTT GCAATCGTGA AAGACCCCGT TCTGAACAAA GACAACAGCA ACCCGTGGGA 360

ACAGGATTCC GTGGAGATCT TCATCGACGA GAACAACCAC AAGACAGGAT ACTACGAAGA 420

CGACGACGCA CAGTTCAGGG TGAACTACAT GAACGAGCAG ACGTTTGGAA CGGGAGGAAG 480

TCCAGCGAGG TTCAAGACAG CGGTGAAACT GATCGAAGGA GGATACATAG TTGAGGCAGC 540

GATCAAGTGG AAGACGATCA AACCCACACC GAACACGGTG ATAGGATTCA ACATCCAGGT 600

GAACGATGCG AACGAGAAAG GGCAGAGGGT CGGTATCATC TCCTGGAGCG ATCCCACAAA 660

CAACAGCTGG AGAGATCCTT CAAAGTTCGG TAACCTCAGG CTCATCAAGG GATCTGGTCC 720

GACCCCATCC CCAACGCGTA AGCTCTGCAG CCTGGACAAC GGGGACTGTG ACCAGTTCTG 780

CCACGAGGAA CAGAACTCTG TGGTGTGCTC CTGCGCCCGC GGGTACACCC TGGCTGACAA 840

CGGCAAGGCC TGCATTCCCA CAGGGCCCTA CCCCTGTGGG AAACAGACCC TGGAACGCAG 900

GAAGAGGTCA GTGGCCCAGG CCACCAGCAG CAGCGGGGAG GCCCCTGACA GCATCACATG 960

GAAGCCATAT GATGCAGCCG ACCTGGACCC CACCGAGAAC CCCTTCGACC TGCTTGACTT 1020

TGATCAGACG CAGCCTGAGA GGGGCGACAA CAACATTGAA GGTCGTATCG TGGGAGGCCA 1080

GGAATGCAAG GACGGGGAGT GTCCCTGGCA GGCCCTGCTC ATCAATGAGG AAAACGAGGG 1140

TTTCTGTGGT GGAACCATTC TGAGCGAGTT CTACATCCTA ACGGCAGCCC ACTGTCTCTA 1200

CCAAGCCAAG AGATTCAAGG TGAGGGTAGG GGACCGGAAC ACGGAGCAGG AGGAGGGCGG 1260

TGAGGCGGTG CACGAGGTGG AGGTGGTCAT CAAGCACAAC CGGTTCACAA AGGAGACCTA 1320

TGACTTCGAC ATCGCCGTGC TCCGGCTCAA GACCCCCATC ACCTTCCGCA TGAACGTGGC 1380

GCCTGCCTGC CTCCCCGAGC GTGACTGGGC CGAGTCCACG CTGATGACGC AGAAGACGGG 1440

GATTGTGAGC GGCTTCGGGC GCACCCACGA GAAGGGCCGG CAGTCCACCA GGCTCAAGAT 1500

GCTGGAGGTG CCCTACGTGG ACCGCAACAG CTGCAAGCTG TCCAGCAGCT TCATCATCAC 1560

CCAGAACATG TTCTGTGCCG GCTACGACAC CAAGCAGGAG GATGCCTGCC AGGGGGACAG 1620

CGGGGGCCCG CACGTCACCC GCTTCAAGGA CACCTACTTC GTGACAGGCA TCGTCAGCTG 1680

GGGAGAGGGC TGTGCCCGTA AGGGGAAGTA CGGGATCTAC ACCAAGGTCA CCGCCTTCCT 1740

CAAGTGGATC GACAGGTCCA TGAAAACCAG GGGCTTGCCC AAGGCCAAGC CTACTAGTCA 1800

TGATGAACTT TAAGAGCTCC AGCTTTTGTT CCCTTTAGTG AGGGTTAATT GCGCGCTTGG 1860

<210> 22

<211> 597

<212> PRT

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:construct translation

<400> 22

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  1               5                  10                  15

Leu Phe Ser Pro Pro Ile Tyr Ser Ala Gly His Asp Tyr Arg Asp Ala
             20                  25                  30

Leu Arg Lys Thr Ser Ala Ser Gly Ile Met Val Ala Thr Ala Lys Tyr
         35                  40                  45

Gly Thr Pro Val Ile Asp Gly Glu Ile Asp Glu Ile Trp Asn Thr Thr
     50                  55                  60

Glu Glu Ile Glu Thr Lys Ala Val Ala Met Gly Ser Leu Asp Lys Asn
 65                  70                  75                  80

Ala Thr Ala Lys Val Arg Val Leu Trp Asp Glu Asn Tyr Leu Tyr Val
                 85                  90                  95

Leu Ala Ile Val Lys Asp Pro Val Leu Asn Lys Asp Asn Ser Asn Pro
            100                 105                 110

Trp Glu Gln Asp Ser Val Glu Ile Phe Ile Asp Glu Asn Asn His Lys
        115                 120                 125

Thr Gly Tyr Tyr Glu Asp Asp Asp Ala Gln Phe Arg Val Asn Tyr Met
    130                 135                 140

Asn Glu Gln Thr Phe Gly Thr Gly Gly Ser Pro Ala Arg Phe Lys Thr
145                 150                 155                 160

Ala Val Lys Leu Ile Glu Gly Gly Tyr Ile Val Glu Ala Ala Ile Lys
                165                 170                 175

Trp Lys Thr Ile Lys Pro Thr Pro Asn Thr Val Ile Gly Phe Asn Ile
            180                 185                 190

Gln Val Asn Asp Ala Asn Glu Lys Gly Gln Arg Val Gly Ile Ile Ser
        195                 200                 205

Trp Ser Asp Pro Thr Asn Asn Ser Trp Arg Asp Pro Ser Lys Phe Gly
    210                 215                 220

Asn Leu Arg Leu Ile Lys Gly Ser Gly Pro Thr Pro Ser Pro Thr Arg
225                 230                 235                 240

Lys Leu Cys Ser Leu Asp Asn Gly Asp Cys Asp Gln Phe Cys His Glu
                245                 250                 255

Glu Gln Asn Ser Val Val Cys Ser Cys Ala Arg Gly Tyr Thr Leu Ala
            260                 265                 270

Asp Asn Gly Lys Ala Cys Ile Pro Thr Gly Pro Tyr Pro Cys Gly Lys
        275                 280                 285

Gln Thr Leu Glu Arg Arg Lys Arg Ser Val Ala Gln Ala Thr Ser Ser
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290 295 300

Ser Gly Glu Ala Pro Asp Ser Ile Thr Trp Lys Pro Tyr Asp Ala Ala
305 310 315 320

Asp Leu Asp Pro Thr Glu Asn Pro Phe Asp Leu Leu Asp Phe Asp Gln
325 330 335

Thr Gln Pro Glu Arg Gly Asp Asn Asn Ile Glu Gly Arg Ile Val Gly
340 345 350

Gly Gln Glu Cys Lys Asp Gly Glu Cys Pro Trp Gln Ala Leu Leu Ile
355 360 365

Asn Glu Glu Asn Glu Gly Phe Cys Gly Gly Thr Ile Leu Ser Glu Phe
370 375 380

Tyr Ile Leu Thr Ala Ala His Cys Leu Tyr Gln Ala Lys Arg Phe Lys
385 390 395 400

Val Arg Val Gly Asp Arg Asn Thr Glu Gln Glu Glu Gly Gly Glu Ala
405 410 415

Val His Glu Val Glu Val Val Ile Lys His Asn Arg Phe Thr Lys Glu
420 425 430

Thr Tyr Asp Phe Asp Ile Ala Val Leu Arg Leu Lys Thr Pro Ile Thr
435 440 445

Phe Arg Met Asn Val Ala Pro Ala Cys Leu Pro Glu Arg Asp Trp Ala
450 455 460

Glu Ser Thr Leu Met Thr Gln Lys Thr Gly Ile Val Ser Gly Phe Gly
465 470 475 480

Arg Thr His Glu Lys Gly Arg Gln Ser Thr Arg Leu Lys Met Leu Glu
485 490 495

Val Pro Tyr Val Asp Arg Asn Ser Cys Lys Leu Ser Ser Ser Phe Ile
500 505 510

Ile Thr Gln Asn Met Phe Cys Ala Gly Tyr Asp Thr Lys Gln Glu Asp
515 520 525

Ala Cys Gln Gly Asp Ser Gly Gly Pro His Val Thr Arg Phe Lys Asp
530 535 540

Thr Tyr Phe Val Thr Gly Ile Val Ser Trp Gly Glu Gly Cys Ala Arg
545 550 555 560

Lys Gly Lys Tyr Gly Ile Tyr Thr Lys Val Thr Ala Phe Leu Lys Trp
565 570 575

Ile Asp Arg Ser Met Lys Thr Arg Gly Leu Pro Lys Ala Lys Pro Thr
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Ser His Asp Glu Leu
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<210> 23

<211> 28

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 23

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<210> 24

<211> 29

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 24

AAAAGGATCC CTTCTGGTTT TTCGTCAAC 29

<210> 25

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 25

AAAACCCGGG ATGGCGCGAA AATC 24

<210> 26

<211> 21

<212> DNA

<213> Artificial sequence

<220>

<223> Description of Artificial Sequence:PCR primer

<400> 26

TGCGTTCCAG GGTCTGTTTC C 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00330

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL : 435/69.1, 320.1, 410, 419, 468; 800/278, 287, 288, 295, 298

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 410, 419, 468; 800/278, 287, 288, 295, 298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, Agricola, Caplus, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GODDIJN et al. Plants As Bioreactors. Trends In Biotechnology. September 1995, Vol. 13, pages 379-387, see whole document.	1-25
Y	GREENWOOD et al. Purification And Processing Of Cellulose-Binding Domain-Alkaline Phosphatase Fusion Proteins. Biotechnol. Bioeng. 1994, Vol. 44, No. 11, pages 1295-1305, especially pages 1296-1303.	1-25
Y	SEEBOTH et al. In-vitro Cleavage Of A Fusion Protein Bound To Cellulose Using The Soluble yscFs (Kex2) Variant. Appl. Micrbiol. Biotech. 1992, Vol. 37, pages 621-625, especially pages 622-624.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
19 OCTOBER 2000

Date of mailing of the international search report
28 NOV 2000

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Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00330

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GRAHAM et al. The pTugA And pTugAS Vectors For High-level Expression Of Cloned Genes In Escherichia coli. Gene. 1995, Vol. 158, pages 51-54, see whole document.	1-14, 22, 24, 25
Y	US 5,670,623 A (SHOSEYOV et al) 23 September 1997, col. 28, line 15 to col. 29, line 35; col. 41, lne 25 to col. 42, line 67.	1-14, 22, 24, 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00330

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12N 5/04, 15/09, 15/62, 15/64, 15/67, 15/82, 15/90; A01H 5/00